Methods to promote reanimation and rehabilitation of forelimb function after spinal cord injury

Aiva Mara Vitrungs Ievins

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2017

Reading Committee:

Chet Moritz, Chair

Farrel (Ric) Robinson

Eric Chudler

Program Authorized to Offer Degree:

Neuroscience

© Copyright 2017

Aiva Mara Vitrungs Ievins

University of Washington

Abstract

Methods to promote reanimation and rehabilitation of forelimb function after spinal cord injury

Aiva Mara Vitrungs Ievins

Chair of the Supervisory Committee:
Chet Moritz, PhD
Departments of Rehabilitation Medicine and Physiology & Biophysics
Graduate Program in Neuroscience

Spinal cord injury is a devastating condition that can severely limit motor and sensory function and make an individual completely dependent on others for care. The effects of spinal cord injury are debilitating, and available treatments have limited effect. Impaired signal transduction, increased inhibitory molecules near the injury site, and cell damage all contribute to poor recovery from spinal cord injury. The following chapters describe experiments that aim to address these three obstacles to restore movement to paralyzed limbs. Electrical stimulation treatment can induce movements of paralyzed limbs, but these treatments have not yet restored normal motor function. A user-controlled stimulation system may improve the effects of spinal cord stimulation by allowing the user to trigger movements relevant to a particular task. In addition, supplementary treatments that mitigate the biological reaction to spinal cord injury may be necessary to achieve lasting improvements in motor function. Chondroitinase treatment can introduce a period of

plasticity in the adult central nervous system and promote sprouting of descending fibers in the injured spinal cord. Electrical stimulation may help to guide sprouting fibers toward motor neuron targets to circumvent the injury. Stem cell treatment can provide cells to replace or help rehabilitate injured cells, and electrical stimulation may help to guide these new cells toward the formation of beneficial motor circuits. This thesis demonstrates the potential of a brain-controlled spinal stimulation device to restore task-related movements, present a novel behavioral task to evaluate motor recovery in a rat model of spinal cord injury, and investigate the utility of chondroitinase and stem cell treatments in restoring function after spinal cord injury.

Table of Contents

Chapter 1: Introduction..**9**
1.1 Abstract 9
1.2 Introduction to therapeutic stimulation 9
1.3 Types of spinal stimulation..11
1.4 Epidural spinal stimulation 12
1.5 Transcutaneous stimulation 16
1.6 Intraspinal stimulation..18
1.7 Challenges..19
1.8 Future directions 20
Chapter 2: Reanimation of forelimb function in a rat model of spinal cord injury..**23**
2.1 Brain-triggered spinal stimulation in a rat model of spinal cord injury 23
Introduction 23
Methods 24
Results 30
Discussion 32
Supplementary materials 32
2.2 Novel behavioral task and nosepoke-triggered spinal stimulation in a rat model of spinal cord injury 34
Introduction 34
Methods 36
Results 44
Discussion 53
Conclusion 55
Supplementary materials 56
Chapter 3: Methods to rehabilitate forelimb function after spinal cord injury 58
3.0 Introduction..58
3.1 Chondroitinase and electrical stimulation as a combined treatment for spinal cord injury 60
Methods..60
Results 63
Discussion..68
3.2 Pluripotent stem cells and electrical stimulation as a combined treatment for spinal cord injury 69

3.2.1 Neural stem cell treatment in a rat model of spinal cord injury 69
Methods 69
Results 71
3.2.2 Combined electrical stimulation and neural stem cell treatment in a rat model of spinal cord injury 75
Methods 75
Results 78
3.3 Discussion 81
Chapter 4: Future Directions 83
4.1 Future Directions 83
References 86

List of Figures

Figure 1.1 Stimulation electrode locations 12

Figure 2.1 Cortical recording array 26

Figure 2.2 Single unit recording during lever press task 27

Figure 2.3 Spinal contusion injury device 28

Figure 2.4 Spinal cord implants 29

Figure 2.5 Brain-triggered spinal stimulation facilitates lever deflection 30

Figure 2.6 Technical limitations affecting experimental outcomes 31

Figure 2.7 Lever arena 39

Figure 2.8 Lever performance variability post-injury, pre-implant 45

Figure 2.9 Lever performance patterns 47

Figure 2.10 Weekly lever performance after injury 49

Figure 2.11 Limb use asymmetry scores 50

Figure 2.12 IBB scores 51

Supplementary Calculation 2.1 Determination of maximum safe stimulation current 55

Supplementary Figure 2.13 Stimulation thresholds 57

Figure 3.1 Limb use asymmetry improvement 64

Figure 3.2 IBB scores 66

Figure 3.3 Movement thresholds of stimulated and unstimulated wires 66

Figure 3.4 Mechanical force of probe at time of paw withdrawal 67

Figure 3.5 Examples of c-4-s expression 68

Figure 3.6 Forelimb reaching task scores 72

Figure 3.7 Forelimb asymmetry scores 73

Figure 3.8 IBB scores 74

Figure 3.9 Transplanted cells in the injured spinal cord 75

Figure 3.10 Effect of spinal implant and stimulation on cell survival and multipotency 79

Figure 3.11 Wire tracks in spinal cord tissue 80

Figure 3.12 Wire tracks in spinal cord tissue 80

Acknowledgements

Several people contributed to the success of this work. Aiva Ievins designed experiments and the new behavior task, trained animals, constructed brain and spinal implants, coded hardware, conducted surgeries, collected data, processed tissue, and analyzed data. The following contributors provided support for one or more experiments. Chet Moritz provided general guidance throughout all of these experiments, editing for this document, and laboratory space and equipment for electrical stimulation experiments. Phil Horner provided guidance, laboratory space and equipment for stem cell experiments. Amanda Fischedick provided animal training and data collection support for all experiments, surgical assistance for stem cell experiments, and tissue processing support for chondroitinase and stem cell experiments. Michael Sunshine provided instruction on electrode array construction, surgical assistance for early stimulation experiments and rehabilitation experiments, and data collection and analysis assistance for the brain-triggered spinal stimulation experiment. Soshi Samejima, Annamarie Lahti, Alice Bosma-Moody, and Ryan Carlson provided animal training and data collection support for electrical stimulation experiments. Eric Martinson fabricated and assembled the structural components of the new behavior task arena. Spencer Boyer provided electrical engineering and electrical component assembly support as well as coding support for the new arena. Mark Wronkiewicz provided coding support for the new arena. Nicole Kwan and Lauren Hood provided tissue analysis support for stem cell experiments. Dan McCloy provided statistics consultation and implementation support for the mixed model described in Chapter 2.

Funding sources for this work included the National Science Foundation Graduate Research Fellowship, the University of Washington Graduate Discovery Fellowship, the Center for Sensorimotor Neural Engineering Graduate Training Program, the Graduate Program in Neuroscience, the Paul G. Allen Family Foundation, the Christopher and Dana Reeve Foundation, and the Craig H. Neilsen Foundation.

Chapter 1. Introduction

This chapter has been accepted for publication in Physiology under the title "Therapeutic stimulation for restoration of function after spinal cord injury" by Aiva Ievins and Chet Moritz.

1.1 Abstract

Paralysis due to spinal cord injury can severely limit motor function and independence. This review summarizes different approaches to electrical stimulation of the spinal cord designed to restore motor function, with a brief discussion of their origins and the current understanding of their mechanisms of action. Spinal stimulation leads to impressive improvements in motor function along with some benefits to autonomic functions such as bladder control. Nonetheless, the precise mechanisms underlying these improvements and the optimal spinal stimulation approaches for restoration of motor function are largely unknown. Finally, spinal stimulation may augment other therapies that address the molecular and cellular environment of the injured spinal cord. The fact that several stimulation approaches are now leading to substantial and durable improvements in function following spinal cord injury provides a new perspectives on the previously 'incurable' condition of paralysis.

1.2 Introduction to therapeutic stimulation

Paralysis due to spinal cord injury affects approximately 282,000 people in the United States (100). Spinal cord injury can lead to paralysis of both the upper and lower extremities, severely limiting activities of daily living. People with tetraplegia (paralysis of upper and lower limbs) cite restoration of hand and arm function as their highest priority for functional recovery (3, 31). People with paraplegia (paralysis of only the lower limbs), cite walking movement as a priority, although notably a lower priority than restoration of autonomic functions (3). While many types of therapy may be prescribed for spinal cord

injury rehabilitation, only a few of the most commonly applied methods have demonstrated reliable effects (48), and most do not completely restore the motor function of the paralyzed limbs.

The application of electrical stimulation to treat spinal cord injury has garnered substantial interest from the research community, as it can enhance the electrical activity of neurons after spinal cord injury, and may help to restore function. The spinal cord is an attractive target for stimulation-based rehabilitative therapies, as interventions at the spinal level can take advantage of preserved motor and sensory neural pathways below the injury. Stimulation of the spinal cord evokes fatigue-resistant movements (54), which are typically difficult to achieve with more distal stimulation sites such as peripheral nerves or muscles (74, 95). Spinal stimulation can also produce complex movements involving multiple muscles and joints, such as those required for walking (53, 91) and reaching and grasping (87, 128, 140).

Several groups have used electrical stimulation to improve limb function in awake, behaving, paralyzed animals. This includes the production of hindlimb stepping movements in paralyzed rats (17, 119), cats (7, 54, 93), and non-human primates (19), as well as forelimb reaching movements in rats (69) and non-human primates (87, 99, 140).

In addition to its somatic effects, spinal stimulation may also benefit the autonomic nervous system. Spinal stimulation during motor training can improve and trigger bladder voiding in animal models (35, 103), and a human case study cited improvements in bladder control, sexual function, and temperature regulation after motor training with spinal stimulation (47). While this review is primarily focused on somatic motor improvements, these broad benefits illustrate that stimulation focused on motor rehabilitation may also confer important autonomic benefits to people with spinal cord injury (3).

Furthermore, targeted electrical stimulation may be useful in directing the rehabilitation of specific motor pathways (30). The spinal cord undergoes neural remodeling after injury, and this remodeling can lead to maladaptive changes in neural pathways that may increase detrimental effects such as pain and spasticity (9, 65). Targeted therapeutic electrical stimulation may guide these remodeling mechanisms toward the formation of functional, rather than maladaptive, neural pathways.

1.3 Types of spinal stimulation

Researchers have identified several methods of spinal cord stimulation for the restoration of movement after paralyzing spinal cord injury. Stimulation can be delivered (1) epidurally, with electrodes on the dorsal surface of the cord above the dura, (2) transcutaneously, with electrodes placed on the skin above the vertebral column, and (3) intraspinally, with stimulating electrodes implanted within the spinal cord [Figure 1.1]. The site of stimulation partly determines the neural pathways activated as well as the stimulation parameters required to elicit the desired result (122). The different approaches to spinal stimulation may also differ in their mechanisms of action; current opinion in the field is that intraspinal stimulation likely activates motor pools as well as intraspinal and propriospinal networks to enable coordinated whole-limb movements (131), while sub-threshold epidural and transcutaneous stimulation may increase the baseline excitability of the spinal cord, thereby enabling movements triggered by inputs that remain intact after spinal cord injury (29).

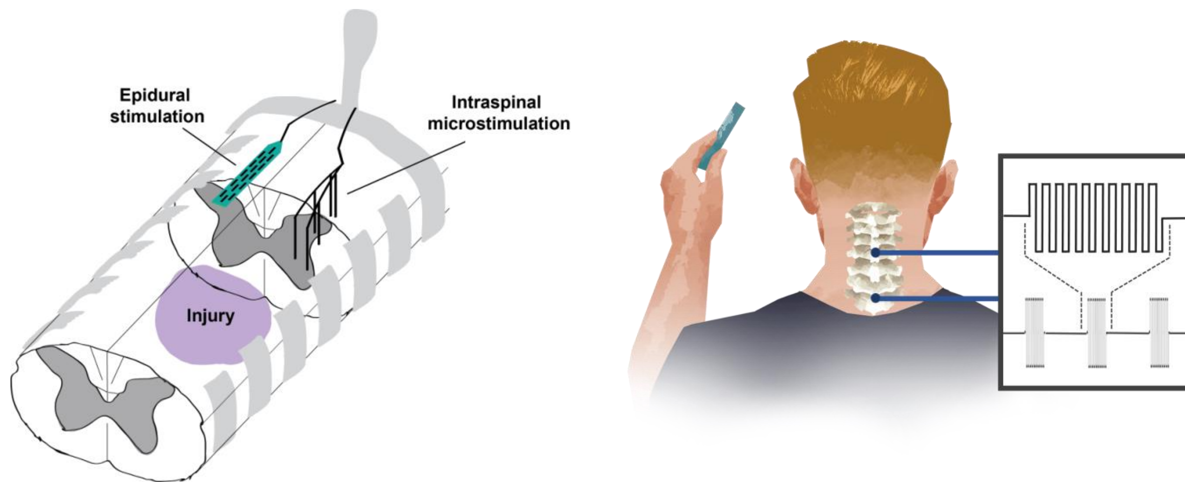


Figure 1.1: Stimulation electrode locations. Illustrations of the location of epidural stimulation compared to intraspinal microstimulation, both applied distal to a contusion injury (left). Stimulation location and stimulation parameters for transcutaneous stimulation applied to the cervical spinal cord utilizing a 10 kHz carrier frequency (right) to improve hand function after spinal cord injury.

1.4 Epidural spinal stimulation

While electrical stimulation treatments for various disorders can be traced back as early as the first century, the utility of spinal cord stimulation for the restoration of motor function is a more recent development. Therapeutic electrical stimulation of the spinal cord first emerged as a treatment for pain described in clinical cases in the late 1960s and early 1970s (26, 123, 124). In a 1967 experiment, Shealy and colleagues stimulated the spinal cord through a single electrode placed on the dura of a man complaining of diffuse chest and abdominal pain. Stimulating at frequencies of 10-50 Hz caused a “buzzing” sensation for the patient, but also eliminated his pain for 5-15 minutes, after which a change in stimulation frequency was required to continue control of the pain (124). This stimulation was believed to inhibit the conduction of pain signals via activating larger sensory fibers in the dorsal columns of the spinal cord and quieting the smaller pain fibers (123). Later experimental results demonstrated effective pain relief using bipolar electrode

arrangements, which allowed for higher frequency (100-200 Hz) stimulation while remaining well below the tissue damage threshold (7.75×10^{-3} W/cm^2) (123).

The clinical applications of spinal cord stimulation quickly expanded, as other groups observed its benefits for improved motor and sensory function in people with multiple sclerosis. Using similar epidural stimulation implants, Cook and Weinstein reported improvements in spasticity, motor function, and sensory function in people with multiple sclerosis treated with epidural spinal stimulation (21). Less than a decade later, Campos and colleagues reported improved motor function and bladder control – among other positive effects – following therapeutic spinal stimulation in people with spinal cord injury as well as those with multiple sclerosis (18). Interestingly, Dimitrijevic and colleagues later observed variable effects of electrical stimulation on relief of spasticity in people with spinal cord injury, citing differences in body position (e.g. standing vs. sitting), differences in spasticity at a given time, and patient-controlled stimulation strength as likely contributors to this variability (27). The same group later outlined standards for epidural stimulation to relieve spasticity, observing that 50-100 Hz stimulation of 2-7V strength and 210 microsecond pulse width worked best. They also noted, however, that the stimulation could be further optimized for each patient by testing different electrode combinations and adjusting stimulus amplitude based on body position (104), highlighting the anatomical variability of spinal pathways similar to those seen between the brains of different people.

Several groups proceeded to refine electrical stimulation of the spinal cord in animal models. They tested the threshold level of stimulation required to evoke movements, then experimented with stimulation that directly evoked movements (supra-threshold). The Skinner group demonstrated that supra-threshold stimulation of the dorsal surface of the spinal cord at a frequency of 3-5 Hz could reliably elicit stepping movements in decerebrated cats (60), and the Edgerton group induced bilateral stepping movements via similar methods in rats, observing that bilateral stepping movements occurred most often

with 40-50 Hz stimulation delivered over the second lumbar spinal segment (56). More recently, the Courtine group observed improved locomotion with supra-threshold epidural stimulation in non-human primates with spinal cord injuries (19).

In contrast to supra-threshold stimulation, sub-threshold stimulation does not immediately evoke movements, but may permit the animal to execute movements in contexts such as treadmill locomotion. For example, the Edgerton group demonstrated that sub-threshold stimulation could induce movement in the presence of proprioceptive inputs, likely by amplifying existing reflexive activity in the rat spinal cord (32).

Parallel findings were observed in human participants. Dimitrijevic's group demonstrated that supra-threshold epidural stimulation at 5-15 Hz could result in lower limb extension in people with complete spinal cord injury and paraplegia. They hypothesized that this effect relied on the activation of primary sensory afferents, which in turn activated a network of neurons within the spinal cord to elicit motor unit activity and muscle contraction (64). Human and animal studies indicate that epidural stimulation of the spinal cord may induce movements either by direct electrical activation of motor or sensory units (56, 60, 64), or by the facilitation, or increase, of baseline motor unit activity (32, 41). Increasing baseline motor unit activity could bring the motor units closer to threshold – the level of activity required to produce a movement. This sub-threshold stimulation technique has garnered substantial interest in the spinal stimulation research community in recent years.

In contrast to direct activation of motor units, epidural stimulation more recently enabled otherwise paralyzed people to make volitional movements in the presence of continuous, sub-threshold stimulation. Recent work has demonstrated the utility of this 'enabling' epidural stimulation for promoting both lower (4, 47) and upper (73) limb movements. These studies show an impressive return of voluntary lower limb movement with epidural stimulation in people with complete and incomplete spinal cord injuries (4, 47), as well as

improved volitional hand control with epidural stimulation in people with motor-limiting cervical spinal cord injuries (73).

For some participants, the benefits of sub-threshold epidural stimulation persist beyond the period of stimulation. The exciting therapeutic benefit was noted by both participants in the upper-extremity study, whose hand function remained improved after stimulation had ceased (73). This encouraging result further supports the need for an evaluation of the precise mechanisms activated by epidural stimulation. Just as customized stimulation parameters such as stimulation frequency and amplitude for individual patients and specific tasks typically lead to better functional improvements during the stimulation period (2, 108, 109, 119), customized parameters and pairing with other rehabilitation strategies geared toward enabling sustained function after stimulation may provide an additional benefit.

The mechanisms responsible for the effect of epidural stimulation on paralyzed limbs have intrigued many researchers. A 1975 review of early work in neural stimulation described the multitude of factors at play, explaining that an understanding of the precise cells and tissues activated by stimulation will require detailed knowledge of the cell and tissue properties, electrode configurations, and stimulus parameters such as waveform shape, duration and magnitude (107). Although the field may yet lack a complete understanding of the complex interactions of these elements, some generalized mechanistic explanations have emerged as described below.

While epidural stimulation activates both afferent and efferent pathways (84, 111), supraspinal and sensory inputs driven by the patient's intent and position may dictate the specific motor units recruited for a given task. For example, it is likely that the sensory signals produced by weight-bearing standing can selectively enhance the activity of relevant motor units during stimulation periods (45, 111), effectively increasing activity in the units required to maintain an upright position. Supraspinal input in the form of volitional, conscious motor commands can control lower limb movement in a supine position in the

presence of epidural stimulation, even for people with clinically motor and sensory complete injuries (4, 45). These results are aligned with the current general view of epidural stimulation as an 'enabling' technology capable of enhancing baseline activity or physiological state of the spinal cord such that additional inputs such as proprioceptive inputs in the case of locomotion may activate the appropriate motor pathways for a given task (29). In one study, all twelve participants with motor complete spinal cord injuries could voluntarily produce electromyographic activity in two independent muscles of the paralyzed limbs, although not sufficient to result in movement (89). This provides evidence of spared pathways passing the spinal cord injury in nearly all persons with clinically complete injuries. These spared pathways may carry signals that could be useful in triggering movements in the presence of spinal stimulation. In most cases, epidural stimulation must be applied to observe benefits (4, 45, 108), and carryover of lower extremity motor benefits after the period of stimulation is limited (108). Nonetheless, benefits to autonomic functions such as bladder, bowel and sexual function persist beyond the period of stimulation in both human (47) and animal subjects (35), suggesting a persistent and beneficial reorganization of spinal neural pathways is possible due to stimulation therapy.

1.5 Transcutaneous stimulation

Both electrical and magnetic stimulation applied to the skin surface can improve motor function after injury. Magnetic stimulation applied over the lumbar spinal cord improves spasticity for up to 24 hours following stimulation (70). Similarly, transcutaneous electrical stimulation applied over the thoracic spinal processes leads to improvement in spasticity and augmented stepping ability during stimulation periods for people with spinal cord injuries (51, 52, 80). The adoption of high frequency electrical stimulation permits the application of higher current transcutaneous stimulation to the skin above the spinal cord with minimal discomfort (135, 136). The 10 kHz carrier frequency [Figure 1.1, right]

permits over 100 mA of current to pass through the skin without painful sensations (38). Such stimulation is capable of activating the lumbar spinal cord both in spinally-intact (39, 42, 112, 114) and injured participants (34, 38, 40). As with epidural stimulation, the effects of transcutaneous stimulation depend on body position. For example, the current required to elicit a movement is greater in prone as compared to standing positions, and the magnitude of the response is highest in supine as compared to standing and prone positions (25). These findings reinforce the context-dependent nature of spinal neural pathways that may be leveraged for therapy.

Several groups are also exploring transcutaneous spinal stimulation applied to the cervical region for improving hand and arm function with promising results. Early results suggest that transcutaneous stimulation may confer similar benefits to those of epidural stimulation (40).

Although the electrodes are positioned further from the spinal cord, the basic mechanisms responsible for the effects of transcutaneous stimulation likely also rely on increasing baseline electrical activity to enable movements induced by remaining volitional motor commands or sensory inputs. The Gerasimenko group recently demonstrated that specific electrode configurations can also contribute to enhanced effects of transcutaneous spinal stimulation (113). By stimulating at two sites in rostro-caudal order, first at the site closer to the head and then at the site further down the spinal cord, they were able to elicit stronger responses than by stimulating at individual locations. They suggest that this may be explained by recruitment of motor neurons via both direct and indirect (e.g. sensory, interneuron) pathways in the rostro-caudal stimulation paradigm (113).

Because transcutaneous approaches do not require surgery, they may be more attractive to some people. This experimental approach, however, is quite new, and optimal application schedules and activities to be performed during stimulation are still being discovered. Although precise parameters used in epidural stimulation are unlikely to translate given the

more distant application of current through the skin, a common theme appears to be the need for intensive therapy and exercise to be performed during the application of spinal cord stimulation in order to realize the full benefits to motor function (4, 33).

1.6 Intraspinal stimulation

Intraspinal stimulation differs from epidural and transcutaneous stimulation in that it delivers electrical current through electrodes implanted within the spinal cord. Thus far, intraspinal stimulation studies in humans are rare, but animal work provides insights into the potential benefits of this approach. Intraspinal stimulation can elicit a wide variety of functionally relevant movements in animal models, including movements required for stepping (44, 54, 71, 94–96, 110). It can also elicit a variety of movements related to reaching and grasping (87, 129, 140). When intraspinal stimulation is applied to the ventral spinal cord, direct activation of motor neurons or ventral root axons can occur, leading to single joint movement. When it is applied to the intermediate lamina of the spinal cord, consensus is that stimulation most likely activates axons and subsequently interneurons, cells within the spinal cord that can in turn activate complex neural pathways and result in coordinated motor patterns. This is because electrical stimulation generally activates fibers of passage rather than cell bodies (107), and interneuron fibers are abundant within the spinal cord. The activated interneurons can then activate the reflex and movement coordination pathways in which they participate, which may lead to coordinated multi-joint movements.

Intraspinal stimulation may be especially useful when paired with physical rehabilitation in experiments geared toward activating specific motor pathways or strengthening synapses – the connections between neurons. Rodent studies have demonstrated lasting forelimb motor improvements after intraspinal stimulation of a specific movement (69, 77), even weeks

after stimulation had ceased (77, 81). These results allude to the potential long-term therapeutic effect of intraspinal stimulation. Pairing intraspinal stimulation with rehabilitative physical training may have added benefits, and specific studies that directly address a combined approach would be useful.

Intraspinal stimulation may also confer more benefits if the user can easily control the stimulation. One way to enable such control would be to use signals that are already present during a particular task, such as a brain signal that occurs when an animal attempts to move. Stimulation controlled by activity-related signals is called activity-dependent stimulation. Activity-dependent stimulation may confer long-term benefits when the time between recording of the activity signal and delivery of stimulation falls within a specific time window (77). Activity-dependent stimulation that takes advantage of this time window can strengthen cortico-cortical (61) and cortico-spinal (98) connections in uninjured animals, and further investigation of the utility of this approach in spinal cord injury rehabilitation is warranted. It would be extremely useful to understand the maximum duration of these changes, and whether they can be extended from days (98) and weeks (77) to months or years.

1.7 Challenges

While the stimulation of the spinal cord at epidural, transcutaneous and intraspinal locations as described above has led to substantial advancements in the field, all of these approaches currently fall short of fully restoring natural movements and achieving long-term rehabilitation. Our understanding of the underlying mechanisms responsible for the effect of exogenous stimulation on biological tissue is incomplete, leading to challenges in translation from animal models to humans (22) and difficulty facilitating motor improvements that persist beyond the period of stimulation. Additionally, while epidural stimulation benefits from widespread clinical acceptance due to its long history as a pain treatment, translation

of intraspinal stimulation methods will likely take more time, as development of hardware and novel surgical and application techniques is still underway.

Nonetheless, electrical stimulation shows therapeutic potential in the treatment of spinal cord injury motor deficits, and investigations of the underlying mechanisms and optimal stimulation parameters will continue to drive progress toward restoring natural movements to paralyzed limbs.

1.8 Future directions

While promising early results of electrical spinal stimulation indicate a prominent role in enhancing motor recovery, the potential of this technology to elicit long-term, sustained improvements will most likely require further refinement and perhaps a combination of multiple treatment approaches. Such approaches are referred to as combinatorial treatments, as they include a combination of interventions.

Such combinatorial interventions might target the molecular environment of the injured spinal cord to further increase its excitability and enhance the effects of therapeutic electrical stimulation. For example, pharmacological agents that increase excitability, such as serotonergic agonists or inhibitory neurotransmitter antagonists, appear to enhance the effects of epidural stimulation (16, 43). These pharmacological treatments can in some cases enhance the effects of locomotor training (30) and epidural stimulation (23). When administered orally during a period of transcutaneous stimulation treatment in humans, the serotonergic agonist buspirone enhanced motor function during and beyond acute stimulation treatments (40). This is consistent with prior results in animals, in which quipazine, another serotonergic agonist, appeared to regulate the stepping rhythm induced by epidural stimulation (41). Combinatorial approaches that employ multiple tools may prove to be the most useful. For example, combinations of low-dose pharmacological agents, electrical stimulation, and motor training have demonstrated functional

improvements in animal models of spinal cord injury (16, 28, 30). The combinations of stimulation and pharmacological approaches are reviewed in detail elsewhere (30, 41).

In addition to pharmacological agents that directly affect spinal cord excitability, agents that enhance the plasticity of the spinal cord may also improve motor outcomes. These agents typically interfere with molecular pathways that inhibit plasticity. For example, approaches that interfere with Nogo-A, a component of myelin that inhibits neurite outgrowth, can enhance cerebrospinal tract sprouting and improve hindlimb locomotion after spinal cord injury (117). Similarly, dissolution of chondroitin sulfate proteoglycans (CSPGs), extracellular matrix components that limit synapse formation (15), has resulted in sprouting of ascending and descending neural projections and improved motor function (15, 62, 126). Interestingly, a combination of anti-Nogo and enzymatic treatment to dissolve CSPGs yielded greater improvements in motor function than either treatment alone (138). Ongoing and future work testing the combination of electrical spinal stimulation and these plasticity-promoting interventions is a promising avenue to improve function after spinal cord injury.

While pharmacological interventions may promote excitability and plasticity in the cells that remain viable after spinal cord injury, replacing damaged cells may further enhance electrical stimulation treatments. Spinal cord injury often results in cellular damage and demyelination or dysmyelination, whereby the insulating material that enables efficient electrical conduction through axons is lost or damaged. Stem cells might promote the repair of this damage; for example, neural and glial cells derived from transplanted neural stem and progenitor cells (90, 101, 133) promote remyelination of axons near the injury site and promote motor improvement after spinal cord injury (24, 50). However, because stem cells may mature into many different cell types, it is important to carefully direct the cell toward a specific type, or fate, prior to transplant to achieve optimal results (50). Stem cell and neural progenitor treatments also improve reaching performance and hand function (102, 115).

Intriguingly, stem cells might also respond to therapeutic electrical stimulation, potentially by migrating toward the site of injury and providing support to damaged neurons, or by forming new neural networks to bridge the gaps caused by spinal cord injury. Early results from studies investigating this combination are promising. Electrical stimulation appears to promote transplanted cell survival after peripheral nerve axotomy *in vivo* (46), indicating the potential for a positive interaction of the two approaches. Additionally, the application of electrical current can affect neural stem cell migration *in vitro* (5, 85, 137). Based on these findings, perhaps electrical stimulation could be used to guide stem cells toward sites of cellular damage *in vivo*.

Taken together, these currently disparate approaches suggest many potential avenues for combined therapeutic electrical stimulation, cell-based, and pharmacological therapies in future work. The early successes of electrical stimulation therapies are encouraging, but restoring complete function may require the combination of many approaches that address the multi-faceted effects of spinal cord injury. It is an exciting time in the field of spinal cord injury, as future studies have a multitude of potentially viable treatment options to explore. Going forward, careful and systematic evaluation of therapeutic stimulation approaches and their combinations with molecular and cellular interventions may be necessary to deliver effective new treatments to benefit people with spinal cord injuries.

Chapter 2. Reanimation of forelimb function in a rat model of spinal cord injury

2.1 Brain-triggered spinal stimulation in a rat model of spinal cord injury

Introduction

Electrical stimulation of the spinal cord is a promising tool that can both directly induce and indirectly enable the movement of paralyzed limbs after spinal cord injury. Spinal stimulation can evoke complex movements in paralyzed limbs after spinal cord injury, such as those required for reaching and grasping (87, 140). Stimulating the spinal cord can also evoke fatigue-resistant movements and smooth force grading, as it recruits motor units in their natural recruitment order (54). Previous work has demonstrated the utility of intraspinal microstimulation in restoring locomotor movements after spinal cord injury in cats (54, 92, 94), and more recently intraspinal microstimulation (ISMS) has evoked a variety of forelimb movements in primates (87, 122) and rats (128).

Intraspinal microstimulation can thus generate naturalistic movements in paralyzed limbs after spinal cord injury, but it may be more effective when triggered by natural stimuli, such as relevant task-based signals or brain activity. People with paralysis due to stroke or spinal cord injury can modulate their brain signals to control a robotic arm (20, 49); similar signals combined with spinal stimulation might help to restore movement to paralyzed limbs. Brain activity used to trigger functional electrical stimulation of the muscles results in improved motor control after spinal cord injury in non-human primates (86). Recent work has also demonstrated the utility of brain controlled muscle stimulation in restoring functional movements in humans (1, 14). Thus, it is feasible that brain activity could also trigger intraspinal microstimulation to restore functional movements to paralyzed limbs.

Using brain activity to trigger ISMS may also promote rehabilitation of the spinal cord. When neural activity recorded in one area of cerebral cortex triggers stimulation in another, the connection between the two is strengthened (61), likely due to mechanisms of Hebbian plasticity (88). Hebb's postulate, commonly summarized as "neurons that fire together, wire together," states that neurons whose activity is synchronized in time are more likely to establish a stronger connection. This phenomenon depends on the temporal relationship of the two neurons' action potentials (11). If stimulation is delivered within a specific time window after the detection of brain activity (11), brain activity-dependent stimulation of the spinal cord results in strengthening of a healthy corticospinal synapse (98). Brain activity-dependent stimulation of the injured spinal cord might similarly result in the strengthening of corticospinal networks that enable movement of the paralyzed limbs.

Here, we investigate the feasibility of a brain-controlled intraspinal stimulation paradigm for the restoration of forelimb function in a rat model of spinal cord injury. We use single-unit cortical recordings to trigger functional intraspinal microstimulation and evoke movements in real time during a motor behavior task. We demonstrate the feasibility of this paradigm and discuss several technical challenges pertaining to this method. We also suggest solutions to those challenges to enable further investigation, and present a detailed solution that addresses two of those challenges in section 2.2.

Methods

Subjects

All experiments were conducted on adult female Long Evans rats (>250 g). All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Behavioral training

We trained animals to proficiency in a lever press task. The task required them to reach out of a window cut in a clear acrylic arena, in a manner similar to that required for the standard pellet reaching task (75, 76). In this task, instead of reaching for a small chocolate-flavored food pellet on a block outside the arena, the animals reached for and depressed a lever affixed to a flex sensor. A photo sensor interfaced with an RZ5 processor (Tucker Davis Technologies) and positioned below the window just outside the arena detected when the animal's hand broke the plane of the arena, and we counted this as a lever press attempt. The flex sensor interfaced with the RZ5 processor to detect when the flex data crossed a pre-determined threshold. Once the animal pressed the lever to this threshold, the system would trigger the release of a pellet from a pellet dispenser located at the rear of the arena. The animal would retrieve and eat the pellet, and after a brief time-out period could access the lever again. We defined proficiency as 70% or greater successful lever presses out of all lever press attempts.

<u>Brain implant</u>

After the animals reached proficiency on the lever pressing task, we implanted them with custom-built 16-channel tungsten microwire arrays [Figure 1.1] inserted 1.5 mm into the brain in the rat forelimb area. Briefly, our animals were anesthetized with isoflurane (1-3% in 100% oxygen, inhaled), and we performed a craniotomy over the forelimb motor area contralateral to the dominant forelimb used in the lever press task. We inserted the microwire array 1.5 mm deep to target the large Layer V corticospinal neurons. We implanted seven skull screws adjacent to the cranial ridge and wrapped a single ground wire around one of these skull screws to serve as a current return. We secured the implant to the skull screws with dental acrylic.

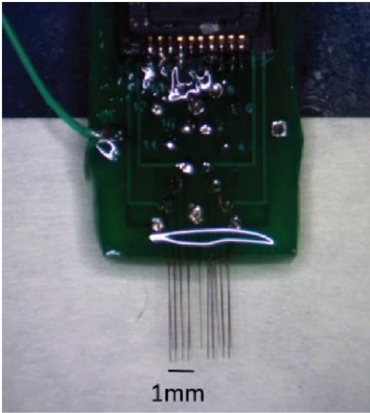


Figure 2.1. Cortical recording array. Cortical arrays consisted of 16 tungsten microwires (30 micron diameter) arranged in two rows of 8 wires each implanted 1.5 mm deep in the forelimb area of the cortex contralateral to the limb used for the lever task. The implant was anchored with a single ground wire (shown here in green) wrapped around a skull screw to serve as a current return.

Post-implant training

After one week of post-surgical recovery, we re-introduced the animals to the lever press task. We recorded their cortical activity using a PZ2 amplifier (Tucker Davis Technologies) and isolated their single unit activity at the beginning of each session using dual time-amplitude windows chosen based on visualization of the amplified cortical activity in real time. For the purposes of these recordings, we defined a single unit as a unique waveform on a single channel that we could distinguish from noise or other unit waveforms using time-amplitude windows. We recorded the animals' single unit activity during the lever press task, and identified units that modulated their firing rates prior to the lever press offline using Matlab (Mathworks) [Figure 2.2]. We trained animals with reliable single-unit recordings for several sessions to identify channels with consistent single unit activity related to the lever press.

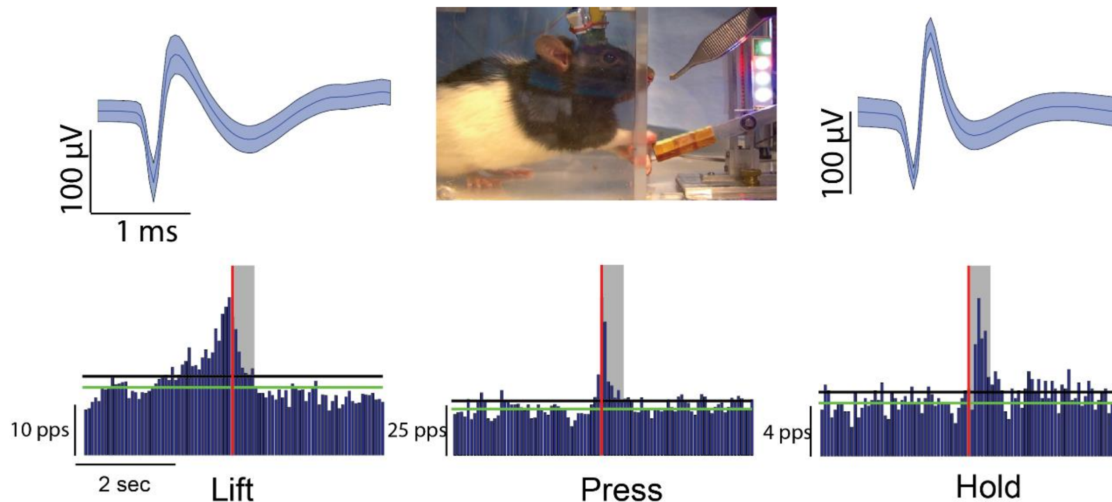


Figure 2.2. Single-unit recording during lever press task. Animals reached out of a window in the front of the arena to press a lever while their cortical activity was recorded (top, middle). Single-unit waveforms (top, left and right) were recorded using dual time-amplitude windows to select action potential phases in a 2.5-millisecond time window. Plots show the mean waveform +/- 1 standard deviation. Firing rate histograms (bottom row) were analyzed offline after each training session to identify units whose firing rates were modulated relative to the lever press. Representative units show firing rate activity that was likely related to the lifting of the arm preceding lever contact (bottom, left), the initial lever press (bottom, middle), and the hold following the press (bottom, right). Red lines indicate the time of lever press and grey shaded regions show the mean lever hold time. Green lines indicate the baseline firing rate for each unit (when the animal was not pressing the lever), and black lines indicate 1 standard deviation of the baseline rate.

Spinal cord injury

Animals with reliable cortical recordings received a unilateral cervical spinal cord contusion injury. Briefly, animals were deeply anaesthetized using injected ketamine (80 mg/kg, intraperitoneal) and xylazine (12 mg/kg, intraperitoneal), and then received a lateralized contusion injury (0.7 mm displacement, 14 ms dwell time) at spinal segments C4-C5 using a modified Ohio State injury device (81, 127) [Figure 2.3]. Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) twice daily for analgesia.

Figure 2.3. Spinal cord contusion injury device. We produced unilateral spinal cord contusion injuries using a modified Ohio State injury device. To stabilize the vertebral column during the spinal injury, we suspended animals with clamps attached to the horizontal black bar. This device produced contusion injuries with a 2 mm wide probe (white arrow) that pressed 0.7 mm deep into the exposed spinal cord for 14 ms.

Spinal implant

After a one-week period of post-surgical recovery, we re-acclimated the animals to the task and confirmed the presence of identifiable single unit cortical activity. We then implanted the animals with 8-10 channel platinum-iridium microwire stimulating arrays as described previously (69) [Figure 2.4]. Briefly, we anesthetized our animals with isoflurane (1-3% in 100% oxygen, inhaled), and performed hemilaminectomies at spinal segments C6-C7. We cut the dura and inserted the wires 1.2-1.6 mm into the spinal cord to target the forelimb motor pools. We then sutured the dura over the wires to secure them in place. We positioned a reference wire in the surrounding muscle tissue and secured the wire bundled again at T2. We routed the wire bundle under the skin in a protective catheter to a connector fixed to the skull. Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) twice daily for two days following implant for analgesia.

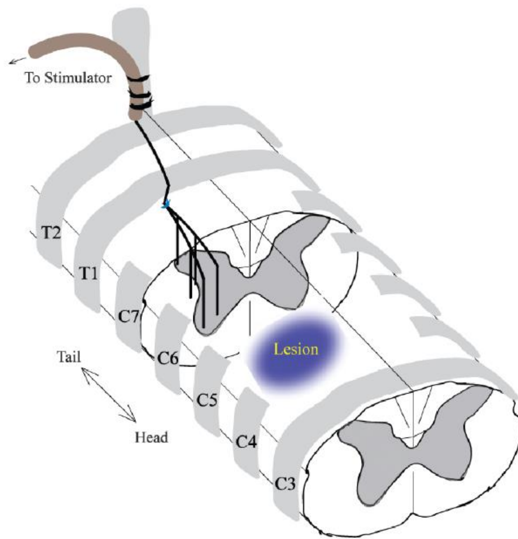


Figure 2.4. Spinal cord implants. Eight to ten polyimide-coated 30-micron platinum-iridium microwires were threaded through a catheter and anchored at the T2 spinal process with a silk suture. A hemi-laminectomy was performed as segments C6-C7 ipsilateral to the spinal cord lesion (blue), and the dura was cut longitudinally. Wires were inserted 1.2-1.6 mm deep to target the ventral motor pools in the grey matter.

<u>Stimulation</u>

Threshold testing

After one week of post-surgical recovery, we stimulated individual channels with single biphasic pulses (300 μsec per phase) to identify channels with forelimb movements. We increased currents at 10 μA intervals from 10 to 500 μA and explored a range of stimulation parameters (3-10 pulses, 50-300 Hz) to identify each individual animal's optimal stimulation channel and settings [Supplementary video 1].

Stimulation of at least one channel typically caused a movement relevant to the lever press task (e.g. elbow extension) that compensated for forelimb function lost after spinal cord injury. We defined optimal settings for the lever press task as those that resulted in an approximate 1cm elbow extension (enough to depress the lever to threshold) without causing discomfort to the animal (e.g. no withdrawal, vocalizations, or excessive grooming of the limb).

Brain-triggered stimulation during lever press task

We continued the study with animals in which we both recorded neural activity related to lever press in the cortex and elicited movement related to lever press by stimulating in the spinal cord. We returned these animals to the lever press task. We chose a single unit in the cortical recordings whose firing rate increased immediately preceding the lever press as the control unit, and a threshold firing rate (typically >3 standard deviations above baseline firing rate) as the control signal for stimulation. When the selected unit's firing rate crossed the specified threshold, the spinal stimulator delivered stimulation on the animals' optimal channel, using the parameters determined during threshold testing. Animals typically completed approximately 30 minutes to 1 hour of training per day, with catch trials interspersed throughout the training session during which the stimulator was turned off to record the animal's unstimulated lever pressing ability.

Results

One animal exhibited improved lever press performance with brain-triggered stimulation, as illustrated by increased lever deflection with the stimulator turned on as compared to lever deflection with the stimulator turned off [Figure 2.5, Supplementary videos 2 and 3].

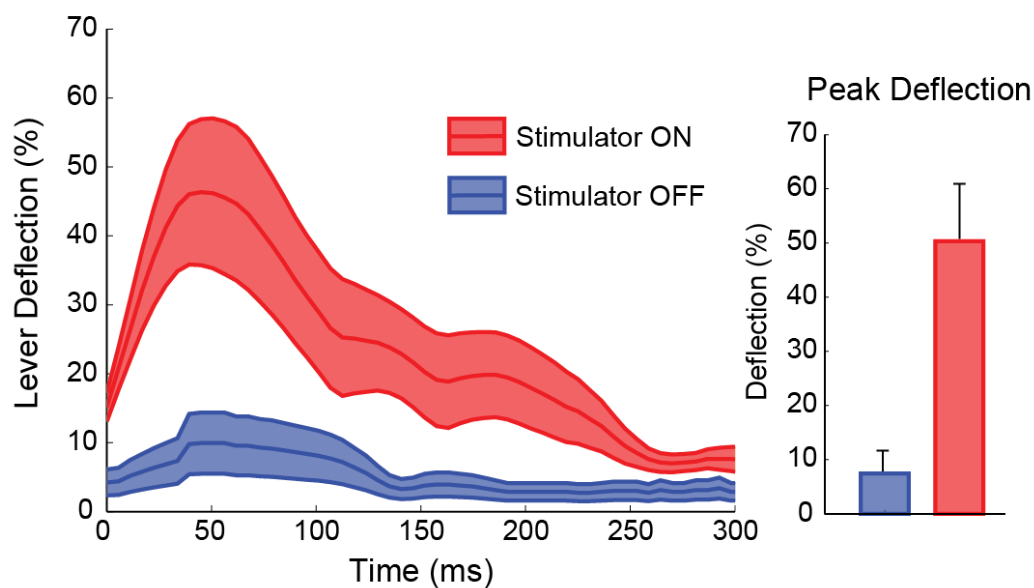


Figure 2.5. Brain-triggered spinal stimulation facilitates lever deflection. Representative lever press trials are shown for a full training session for one animal

(n=60 lever presses, divided among equally interspersed stimulator off and stimulator on trials). Lever deflection plots show mean +/- 1 standard deviation lever deflection. Peak deflection plots show peak + 1 standard deviation lever deflection. Blue = stimulator off, red = stimulator on.

20 subsequent trained animals, however, did not exhibit such improved performance. We attribute this failure to several technical complications [Figure 2.6]. One major problem affecting animals' progression through the experiment was injury variability. Some animals' injury presentations were such that they were unable to lift their forelimbs through the window to reach the lever, while others injuries were mild enough that they were able to press the lever beyond the reward threshold without stimulation. Additionally, several animals' implants failed prior to the final phase of the experiment, such that we lost their cortical signals before the animals reached the spinal stimulation phase of the experiment.

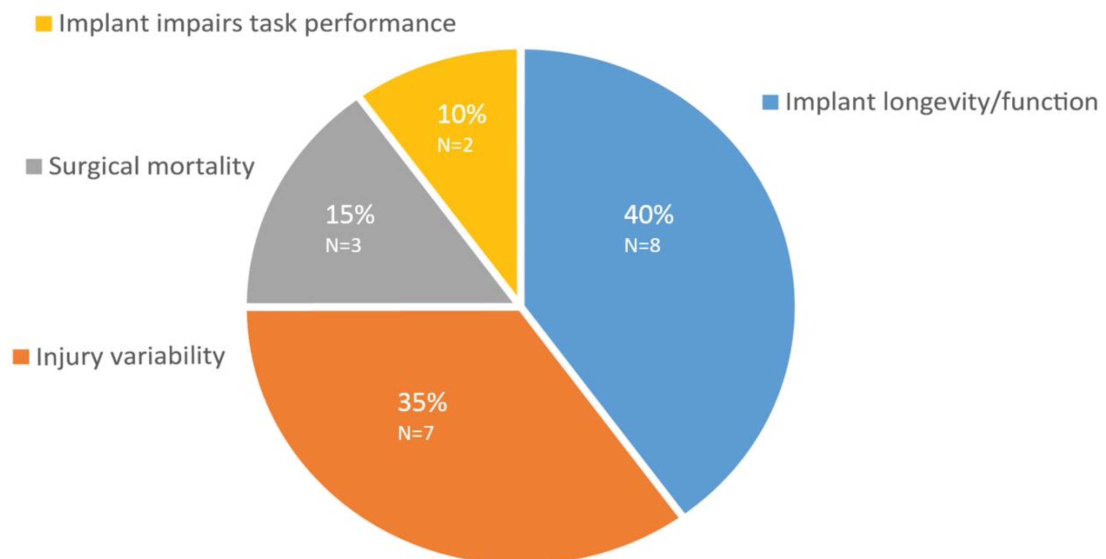


Figure 2.6. Technical limitations affecting experimental outcomes. Issues affecting the experimental outcomes of n=20 animals are categorized above. Major issues include implant longevity/function and injury variability. Surgical mortality (15%) is typical for studies in which multiple surgeries and implants are required. For a small subset of animals, stimulation interfered with task performance, typically because a cortical unit with appropriate signal modulation for the lever press task could not be found.

Discussion

This work demonstrates that it is possible to use brain-controlled spinal stimulation to restore forelimb function after spinal cord injury. It also demonstrates the technical complexity of these experiments and the many variables that must align for this approach to succeed. Namely, it highlights the variability in injury severities and the implant longevity as major obstacles to further development of this method in animal models.

We have modified our methods to try to overcome these obstacles and enable further study of the benefits of brain-controlled spinal stimulation in animal models. We developed a new behavioral task to enable evaluation of animals with a variety of injury severities. This task eliminates many of the problems discovered with the first lever task. In the new task, we place the animals in a more natural position, from which they can press a lever to a specified threshold. The lever automatically moves through fifteen starting positions to sample the entire working space of the animal's forelimb. This allows for the evaluation of animals with different reaching deficits. For this task, we also omitted the cortical recordings, replacing them with a nosepoke trigger for spinal stimulation to enable investigation of the effect of stimulation on lever performance with minimal technical obstacles. We describe this new task and stimulation paradigm in the next section.

Supplementary materials

Supplementary Video 1 (attached file). **Movements evoked by intraspinal microstimulation.** This video shows sample representative movements evoked by stimulation of an implanted ISMS array.

Supplementary Video 2 (attached file). **Brain-controlled intraspinal stimulation during lever press task.** This video shows representative lever press trials with and without brain-triggered spinal stimulation. Clicking sounds correspond to detected single unit activity, tones correspond to the delivery of stimulation. LEDs indicate when the stimulation is available, when the single unit firing rate crosses the threshold for stimulation, and when the animal attempts to press the lever, as defined by its hand passing through the window.

Supplementary Video 3 (attached file). **Brain-controlled intraspinal stimulation during lever press task, half speed.** This video shows the same representative lever press trials as Video 2, with and without brain-triggered spinal stimulation, presented here at half speed to show the movements more clearly. Clicking sounds correspond to detected single unit activity, tones correspond to the delivery of stimulation. LEDs indicate when the stimulation is available, when the single unit firing rate crosses the threshold for stimulation, and when the animal attempts to press the lever, as defined by its hand passing through the window.

2.2 Novel behavioral task and nosepoke-triggered spinal stimulation in a rat model of spinal cord injury

Introduction

Many methods exist to evaluate rat forelimb function after spinal cord injury. Behavioral tests that evaluate forelimb function in awake, behaving animals with spinal cord injuries include tasks that rely on food manipulation, such as two pellet reaching tasks that quantify animals' ability to reach for, grasp, and retrieve a small food pellet (75, 76, 83) and a cereal manipulation task that evaluates their ability to manipulate pieces of cereal with different shapes (58, 59). Tasks that evaluate forelimb function without food manipulation include a vertical exploration task that evaluates animals' paw placement in a cylinder (116), a grooming task (10), a task that evaluates over-ground locomotion (CatWalk Gait Analysis System, Noldus Information Technology), and a task that evaluates locomotion on a horizontal ladder (79). The pellet reaching and cereal manipulation tasks focus on distal and digit function, facilitating the evaluation of animals' food retrieval and manipulation abilities. These tasks enable the evaluation of forelimb movements that the animals would use in their natural environments while foraging or eating. The grooming, vertical exploration, and locomotion tasks are more focused on proximal limb function and weight bearing, and also evaluate common movements that animals' might execute in their natural environment.

There are challenges with each of these methods for some experimental applications. Animals with severe spinal cord injuries have both proximal and distal forelimb function impairments, but distal impairments alone can limit animals' success on tasks that require both distal and proximal forelimb function, even if proximal function is recovered. The pellet reaching tasks evaluate many aspects of distal limb function, but the reward structure is such that animals lacking distal paw function (e.g. those unable to extend their digits to grasp a pellet) are not rewarded. Experiments that require electrical stimulation cables or

other tethers also limit the use of some tasks. The proximal tasks are useful in assessing the gross function of the rat forelimb, but they are not designed to accommodate electrical stimulation based interventions: the grooming task requires access to the animal's head, where a stimulation connector is typically positioned, and the locomotion tasks require a mobile system to allow the animal to remain connected to stimulation cables. Thus, there is a need for a behavioral task that rewards more injured animals to keep them engaged and allows for the incorporation of electrical stimulation hardware such as cables and skull-mounted connectors.

Here, we describe a novel rat behavioral training arena and lever pressing task to address these concerns. It consists of a moveable lever that progresses through 15 starting positions, sampling the working space of the rat forelimb and allowing for the testing of animals with different injury severities. The lever positioning allows the animals to maintain a natural posture, while a nosepoke sensor keeps their bodies in a consistent alignment relative to the lever. The apparatus delivers juice rewards through a tube at the front of the arena.

This behavioral task is also amenable to the testing of electrical stimulation treatments. To demonstrate this, we conducted a stimulation experiment with spinal cord injured rats implanted with intraspinal stimulating electrodes. Although the animals' post-injury performance on the task indicated variable injury severities within the group, their injuries did not prohibit their participation in the task, and their performances indicated that the task covered a range of difficulties, with lever positions closer to the animal resulting in better performance.

We also compared the animals' performance on two standard forelimb function assessments. We evaluated the animals' limb use asymmetry in a standard vertical cylinder exploration task (116) and evaluated their distal limb and digit function using the standard Irvine, Beatties, and Bresnahan (IBB) cereal manipulation task (59). Finally, we evaluated

the effect of several experimental variables on the animals' lever press performance to identify predictors of lever press success.

Methods

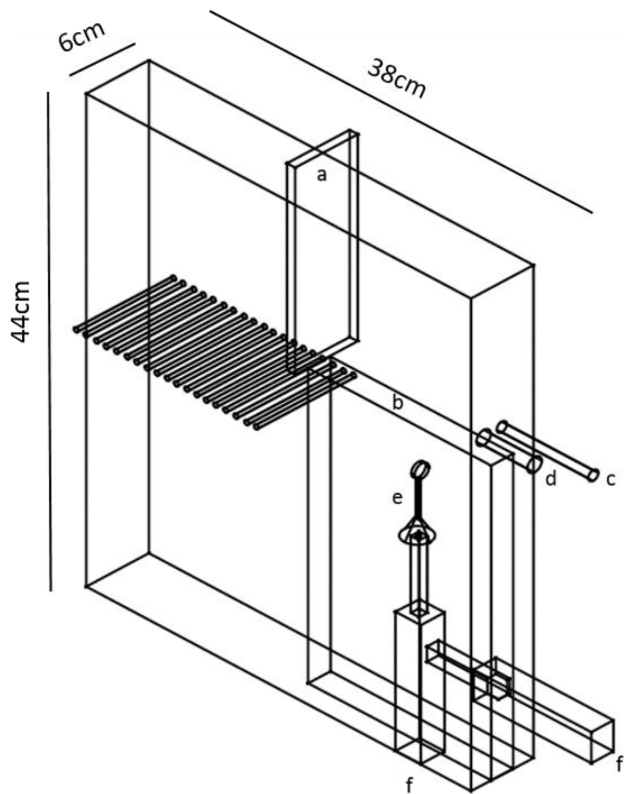
Forelimb range of motion task

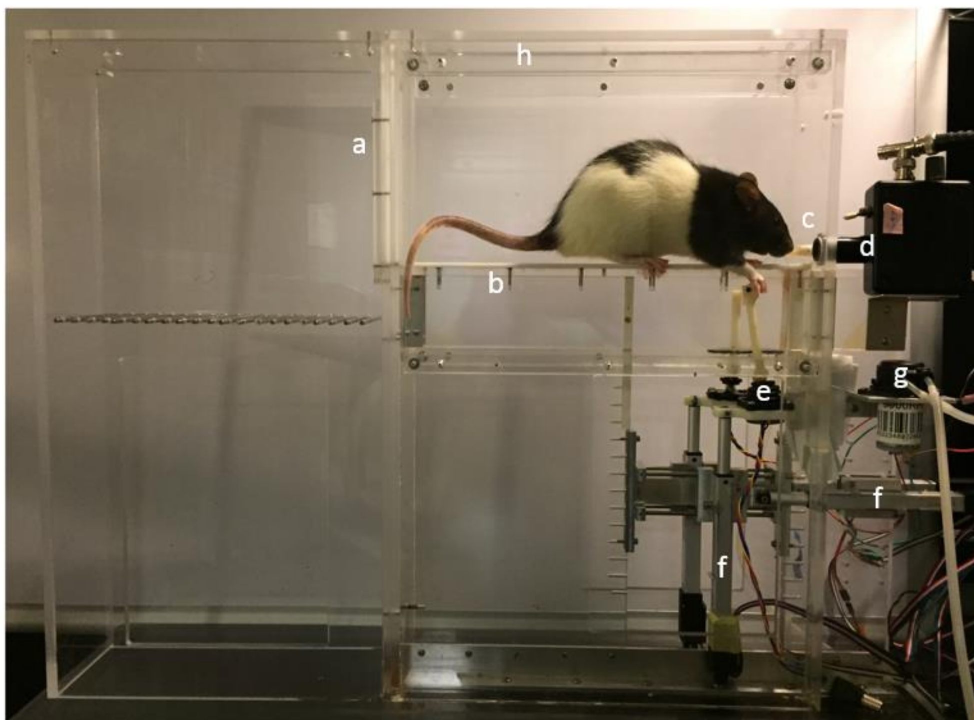
Arena design

The initial prototype of this arena was inspired by the Montoya staircase task, in which animals are positioned on a platform above wells containing small food pellets (83). This arrangement takes advantage of rats' natural foraging behavior, and allows more severely injured animals to participate, as it does not require them to lift their forelimbs through a window in the front of the arena as the Whishaw pellet reaching task requires (76), but rather allows the forelimbs to hang alongside the animal's body in the open space next to the platform. To further enable more severely injured animals that cannot grasp pellets in the Montoya staircase task, we replaced the pellet wells and staircase with a moveable lever that we can adjust to the working range of individual animals' forelimbs, and added a liquid reward system.

Subsequent improvements to the arena included expansion of the rear chamber to create a space for animal acclimation and the addition of a door to keep animals in the front chamber during the task. We also expanded the vertical space and created a track in the arena lid to allow for the attachment and movement of stimulation cables. We elevated the entire arena several inches to allow for the placement of an automatic joystick system consisting of a 3D-printed lever, joystick (Digi-Key Corporation), and 3D-printed joystick mount attached to two linear actuators beneath the platform [Figure 2.7]. We affixed a nosepoke sensor and juice reward system consisting of a 3D-printed sipper tube and small

12V pump to the front of the arena. We connected all digital components to a Raspberry Pi 3 (Adafruit Industries) for sensing, control and data collection.


6cm
38cm
44cm
a
b
c
d
e
f
f


h
a
b
c
d
e
g
f
f

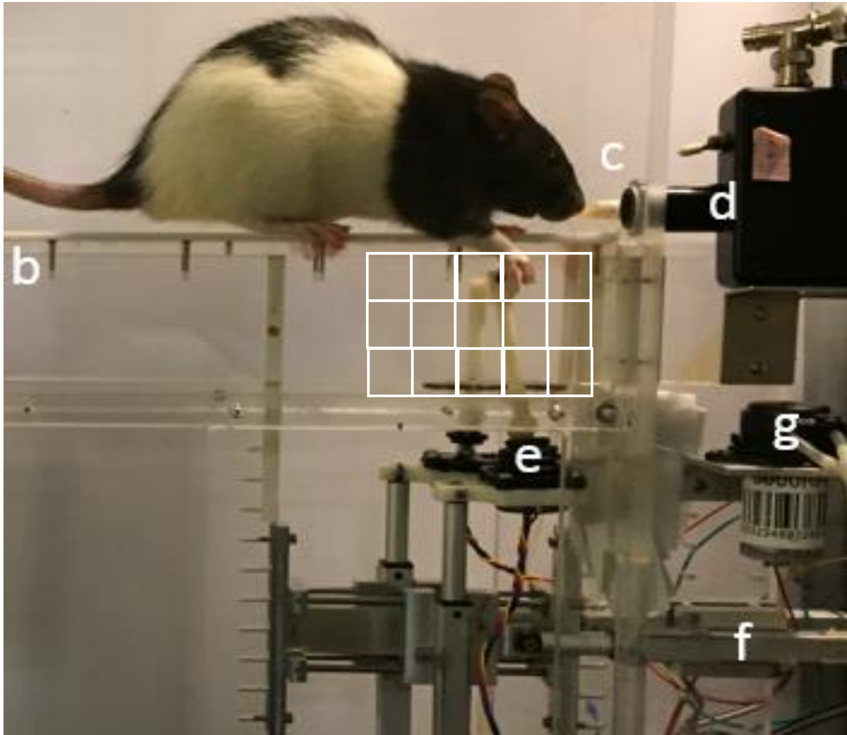


Figure 2.7. Lever arena. A schematic of the arena (top) shows its basic structure, including (a) the wall separating the two chambers, (b) the platform on which the animal rests during the task, (c) the sipper tube that delivers juice rewards to the animal, (d) the nosepoke sensor connection tube, (e) the lever mounted to the joystick, and (f) the linear actuators used to move the lever to each position. Photos of the arena (middle and bottom) show a rat in the arena. The dividing door (a) is closed to keep the animal in the front chamber during the task. The animal rests on the platform (b), while triggering the nosepoke sensor (d) and pressing the lever (e). After the animal completes a successful press, the pump (g) dispenses a drop of apple juice to the sipper tube (c). After the animal completes three successful presses, the linear actuators (f) will move the lever to the next position. The white grid (bottom photo) shows the approximate lever position locations; the rostral edge of each outlined box is the starting lever position at that location. The horizontal linear actuator pushes the vertical actuator along a fixed metal track to maintain alignment when changing lever positions. The top of the arena has transparent horizontal mounts (h) that keep transparent spacer walls in place; these limit the movement of the animal to keep them from exploring the space next to and below the platform. A second lever and second set of linear actuators for testing the other limb are visible in the background.

Lever pressing task

The arena design allows for exploration of forelimb movements in a 2-dimensional 5 cm x 3 cm plane that extends 5 cm horizontally from the front panel of the arena and 3 cm vertically below the rat platform. The current task design consists of 15 lever positions distributed in a 3x5 grid in this space; however, users can easily modify these positions for different applications in the Python script controlling the system.

To promote consistent positioning of the animal's body relative to the lever, we trained animals to trigger and maintain activation of the nosepoke sensor while pressing the lever to a threshold specified in software [Figure 2.7]. The nosepoke sensor acts as a gate for the lever; any lever presses completed without triggering the nosepoke sensor are not counted.

For this study, we set the threshold such that a successful lever press would reach the neutral position of the next lever target (approximately 1cm horizontal deflection toward the rear of the arena). The system rewarded each successful trial with a drop of apple juice delivered via a small pump to a custom sipper tube with a small well at the end. We programmed the lever system to move through the top row of 5 positions first, beginning with the position closest to the animal's nose, and advancing from one position to the next after 3 successful lever presses. After completion of the first row, the lever would move to the most rostral position in the second row, and so on until all positions were tested. If the animal could not successfully deflect the lever to threshold, a trainer manually advanced the lever to the next position using a manual advance button mounted on the control circuit board. Trainers tested each animal on each of the 15 positions, and manually moved the lever after 5 complete failures (arm swing without lever contact) or 5 or more consecutive failed attempts (lever contact insufficient to cross threshold). We trained animals 5 days/week, for 20 minutes each day.

<u>Animal experiments</u>

Subjects

Experiments were conducted on 8 adult female Long Evans rats (>250 g). All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Behavioral training

We trained animals to proficiency in the lever task, with a target performance of 90 successful trials (2 complete circuits). However, the bottom and rear positions proved difficult for some animals even after substantial training, indicating that the lever positions likely reached the bounds of the uninjured rat forelimb range. We also tested animals on the vertical cylinder exploration task (116) (also known as the Limb Use Asymmetry Task, or LUAT), which evaluates their proximal forelimb function by measuring their inclination to bear weight on the injured limb. We calculated LUAT scores by counting the number of wall contacts the animal made with each forelimb when rearing to explore a vertical cylinder and the number of times it landed with each forelimb when returning to a neutral standing position. The number of contacts made with the paralyzed limb divided by the total number of contacts yields the LUAT score. We calculated separate LUAT scores for wall contacts and landings for each animal.

We also tested animals on the Irvine, Beatties, and Bresnahan (IBB) cereal manipulation task (58, 59), which evaluates their distal forelimb function by evaluating their ability to grasp and manipulate cereal of different shapes. The IBB is scored on a 9-point scale, and we tested each animal 3 times on each of two different types of cereal: one spherical and one doughnut-shaped. We calculated mean scores for each animal on each of the two types of cereal.

Spinal cord injury

All animals received a unilateral cervical spinal cord contusion injury as previously described (82). Briefly, we deeply anesthetized animals using injected ketamine (80 mg/kg,

intraperitoneal) and xylazine (12 mg/kg, intraperitoneal). Then each animal received a lateralized contusion injury (0.7 mm displacement, 14 ms dwell time) at spinal segments C4-C5 using a modified Ohio State injury device (82, 127). Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) on the day of injury and twice daily for two days following spinal cord injury for analgesia.

Post-injury training

Following one week of post-surgical recovery, we tested animals again on the lever pressing task, LUAT, and IBB tasks to establish a post-injury, pre-implant baseline. We trained animals on the lever task for 1 acclimation session to re-establish familiarity with the lever task, followed by 3-5 testing sessions each. The LUAT and IBB tasks do not require training, so we tested animals on these tasks only once during this period to establish post-injury, pre-implant baseline performance scores.

Spinal cord implants

We implanted animals with 8-10 channel platinum-iridium microwire stimulating arrays as described previously (69), with slight modifications. We threaded these spinal wires through small holes laser-cut into a thin polyimide backing, and affixed them to the backing with a thin coat of cyanoacrylate. The backing also had notches cut into it, which we used as anchors to suture the implant to the dura. We stripped approximately 0.5 mm of insulation from the wire tips to facilitate stimulation of a broader area and increase the likelihood of reaching the motor pools in the ventral grey matter.

For the surgery, we anesthetized the animals with isoflurane (1-3% in 100% oxygen, inhaled), and performed hemilaminectomies at spinal segments C6-C7. We cut the dura and inserted the wires 1.5-1.7 mm into the spinal cord to target the forelimb motor pools. We anchored the dura to the spinal implant with two sutures. We secured the wire bundled again at T2 with two sutures and routed it under the skin in a protective catheter (19G,

Arrow) to a connector fixed to the skull. We wrapped a ground wire around a skull screw to function as a current return. Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) on the day of implant and twice daily for two days following implant for analgesia.

Spinal stimulation

We stimulated individual channels with single biphasic pulses (300 μ*s* per phase) to identify channels with forelimb movements. We increased currents at 10 μA intervals, starting at 10 μA and increasing until an observable movement occurred. We calculated a maximum safe stimulation current for our electrodes based on published guidelines (78, 121) [Calculation 2.1]. The analyses presented here include data from animals that completed the study with stimulation thresholds below this limit (n=6) [Supplementary Figure 2.13].

On channels with forelimb movements relevant to the lever press (e.g. elbow extension), we performed an additional test using 10 pulses at 50 Hz to produce robust, caudal deflection of the forelimb to assist lever pressing movement. We defined optimal settings for the lever press task as those that resulted in approximately 1 cm elbow extension (enough to displace the lever to threshold) without causing discomfort to the animal (e.g. no withdrawal, vocalizations, or excessive grooming of the limb).

Task-dependent spinal stimulation and continued behavior testing

After one week of post-surgical recovery, we continued testing animals on the lever task 5 days per week. During 3-4 of these daily training sessions each week, animals could trigger spinal stimulation with the nosepoke sensor. This, in turn, triggered a 10-pulse train of 50 Hz stimulation at the pre-determined amplitude in real time. We confirmed stimulation settings prior to each stimulation session, and adjusted amplitudes to ensure that stimulation reliably elicited movements that might assist with the lever deflection toward the threshold position. We used the remaining 1-2 sessions each week as catch trials, during which we connected the animals as usual but turned off the stimulator. This tested

the animals' unstimulated lever pressing abilities. Once per week, we tested the animals on the LUAT and IBB behavioral tests.

Statistics

We used a generalized linear mixed-effects regression model fit by maximum likelihood to evaluate the influence of time, stimulation, stimulation threshold, lever position, LUAT, and IBB scores on lever press performance. We implemented the model in R via a Python Jupyter notebook using the rpy2 package and the lme4 mixed-modeling package. We defined the performance of each animal at each individual lever position as the response variable, with explanatory variables day, stimulation status, stimulation threshold, lever horizontal position, lever vertical position, LUAT wall contact scores, LUAT landing scores, and IBB scores. The lever positions are defined by the linear actuator values, with higher values indicating a more extended actuator; vertical position numbers increase as the vertical actuator pushes the lever closer to the platform, while horizontal values decrease as the lever is pulled closer to the front of the arena. To facilitate model convergence, we converted explanatory variables to comparable scales by normalizing to 0 mean and unit variance. We used only one IBB variable, as the distributions of scores for the two cereal types were nearly identical. Stimulation status remained a binary variable (on vs. off). The number of attempts at each position served as weights. The model was fit using only data following spinal implant to avoid unfair evaluation of the "stimulation off" variable due to higher pre-injury scores.

Results

Injury variability

The variability of our animals' lever performance post-injury, pre-implant indicates variability in the severity of their injuries [Figure 2.8]. Figure 2.8 shows the animals' lever press performance at individual positions as the mean of their daily performances at each

position during testing sessions after injury and before implant. We calculated each animal's overall baseline lever press performance for each day as the mean of its individual position scores for the day, and the mean of these overall performance scores across the days in this post-injury, pre-implant period yielded the mean overall baseline score.

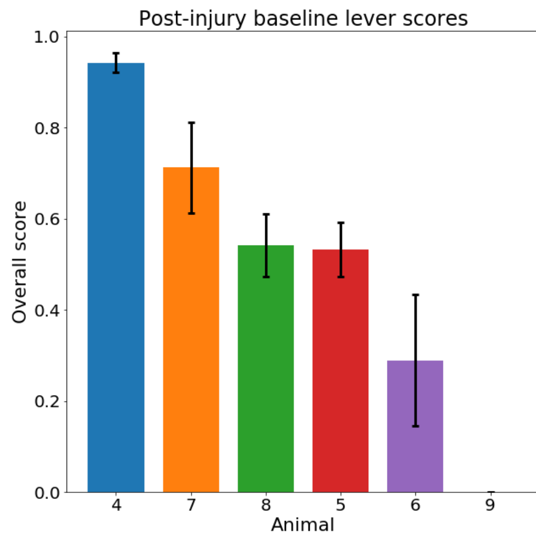
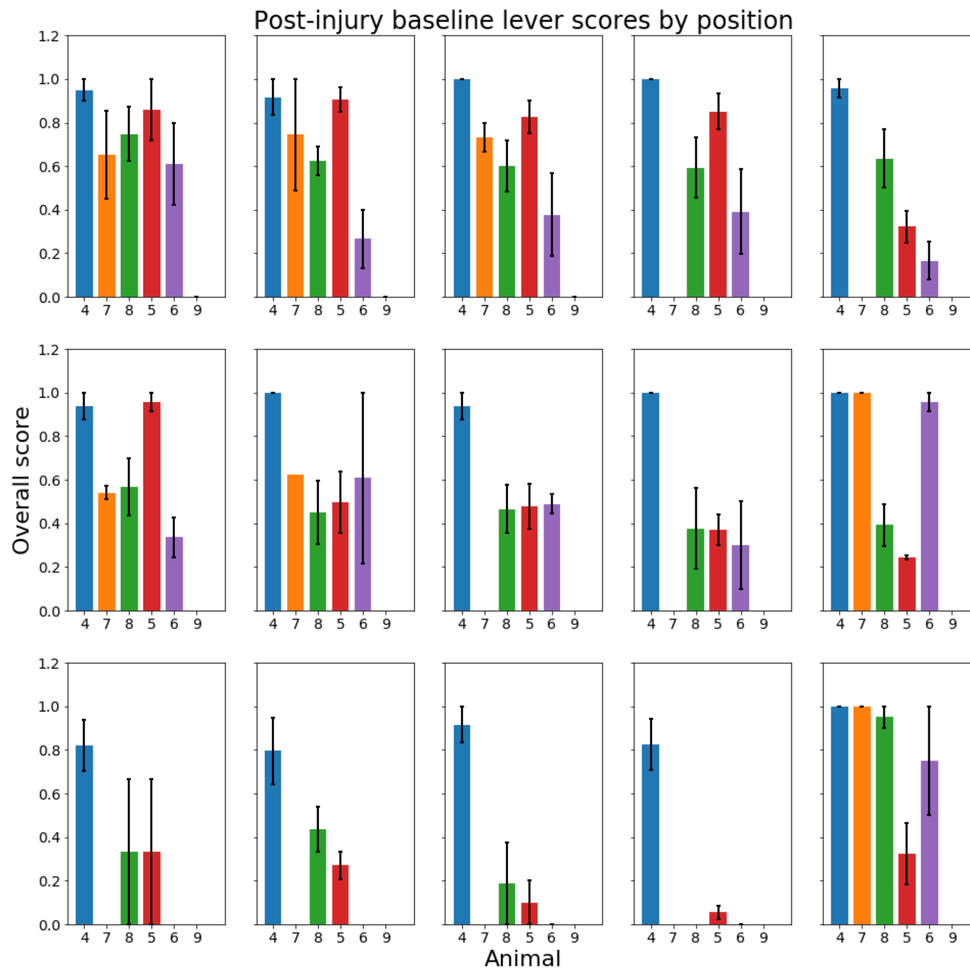


Figure 2.8. Lever performance variability post-injury, pre-implant. Animals' mean overall post-injury, pre-implant lever performance (left) reflects variability among the group. Bars = mean of daily overall performance scores across the post-injury, pre-implant period. Error bars = +/- standard error of the mean. Animals' post-injury, pre-implant performance at individual lever positions (bottom) demonstrates variable performance among animals. Bars = mean daily performance scores at each position across the post-injury, pre-implant period. Error bars = +/- standard error of the mean. The top right plot corresponds to the rostral and proximal lever position in the arena, closest to the nosepoke sensor.



Lever press performance over time

The pattern of the animals' lever press performance at the individual positions changed over time. Heat maps [Figure 2.9] show the animals' mean performance at each lever position before injury and at the conclusion of the study (Week 11). We calculated individual animals' weekly scores as the mean of their daily scores, and used the mean of the animals' weekly scores as the group mean. Darker colors on the heat maps indicate greater success at that position. Before injury [Figure 2.9, left], the animals show some differences in their lever pressing patterns. Example animal 1 has greater lever pressing success at caudal, proximal lever positions than at rostral, distal lever positions, while example animal 2 exhibits greater success at rostral positions. At the conclusion of the study [Figure 2.9,

right], the animals’ group scores show greater success ratios at rostral, proximal positions than at caudal, distal positions, with animals demonstrating some individual differences in this pattern.

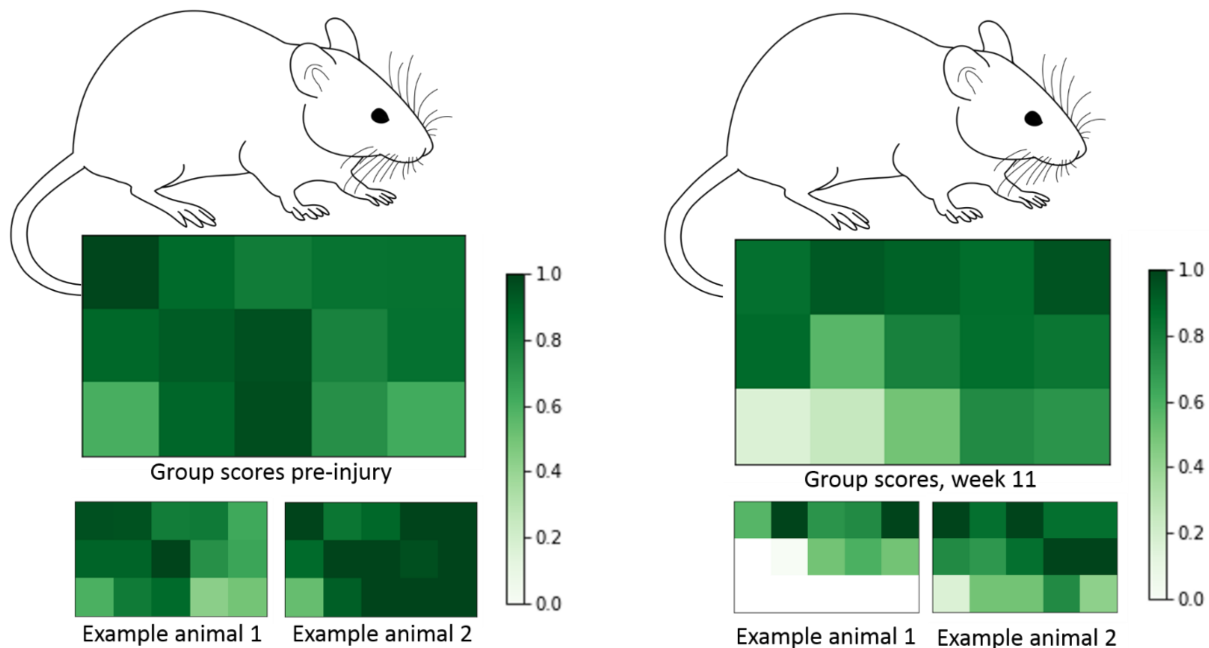
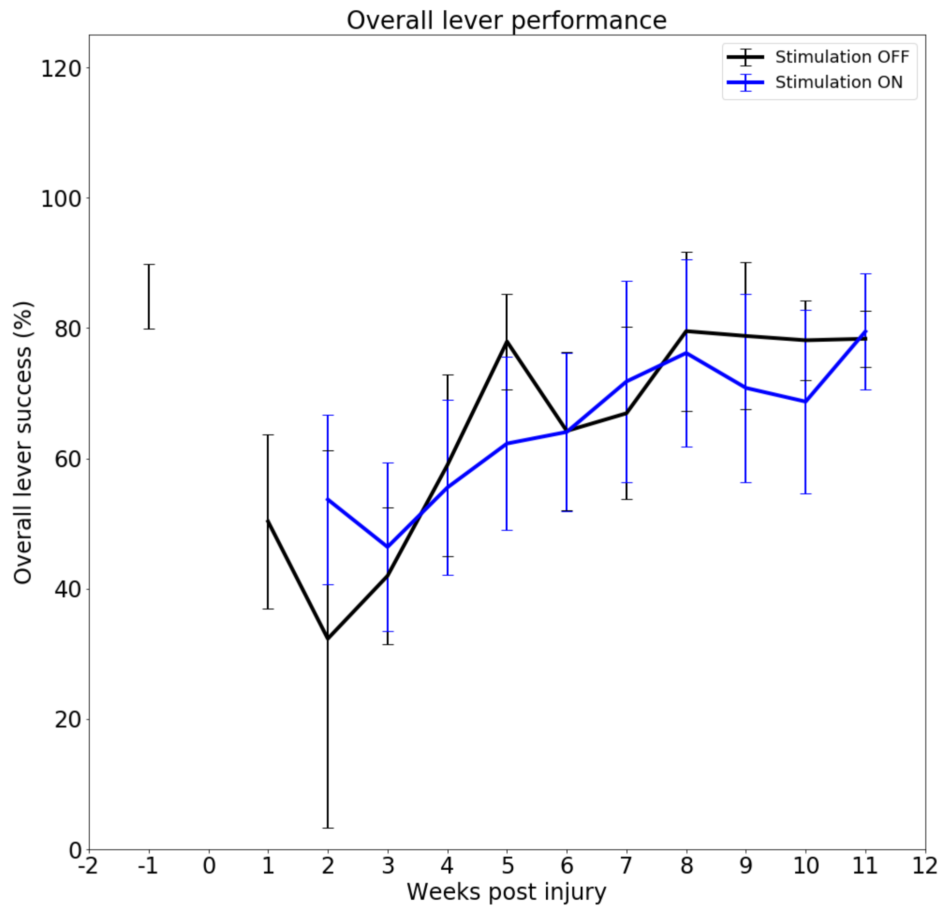


Figure 2.9. Lever performance patterns. The heat maps show the animals’ mean scores at each lever position before injury (left) and at the final week of the study (right). The 15 individual squares correspond to the 15 lever positions, shown in their approximate location relative to the rat (not drawn to scale). Darker shades indicate mean lever success ratios closer to 1, while lighter shades indicate lever success ratios closer to 0.

After implant, animals exhibited improved performance on the lever task over time, both overall and at individual lever positions [Figure 2.10]. We calculated individual animals’ weekly scores as the mean of their daily scores, and used the mean of the animals’ weekly scores as the group mean. There were no clear differences between the stimulation on and stimulation off conditions. Individual lever position performance plots [Figure 2.10, bottom] show the performance trends at each position over time, with proximal positions showing clearer improvement trends and distal positions showing more variable scores.


Overall lever performance
Stimulation OFF
Stimulation ON
Overall lever success (%)
Weeks post injury
0
20
40
60
80
100
120
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12

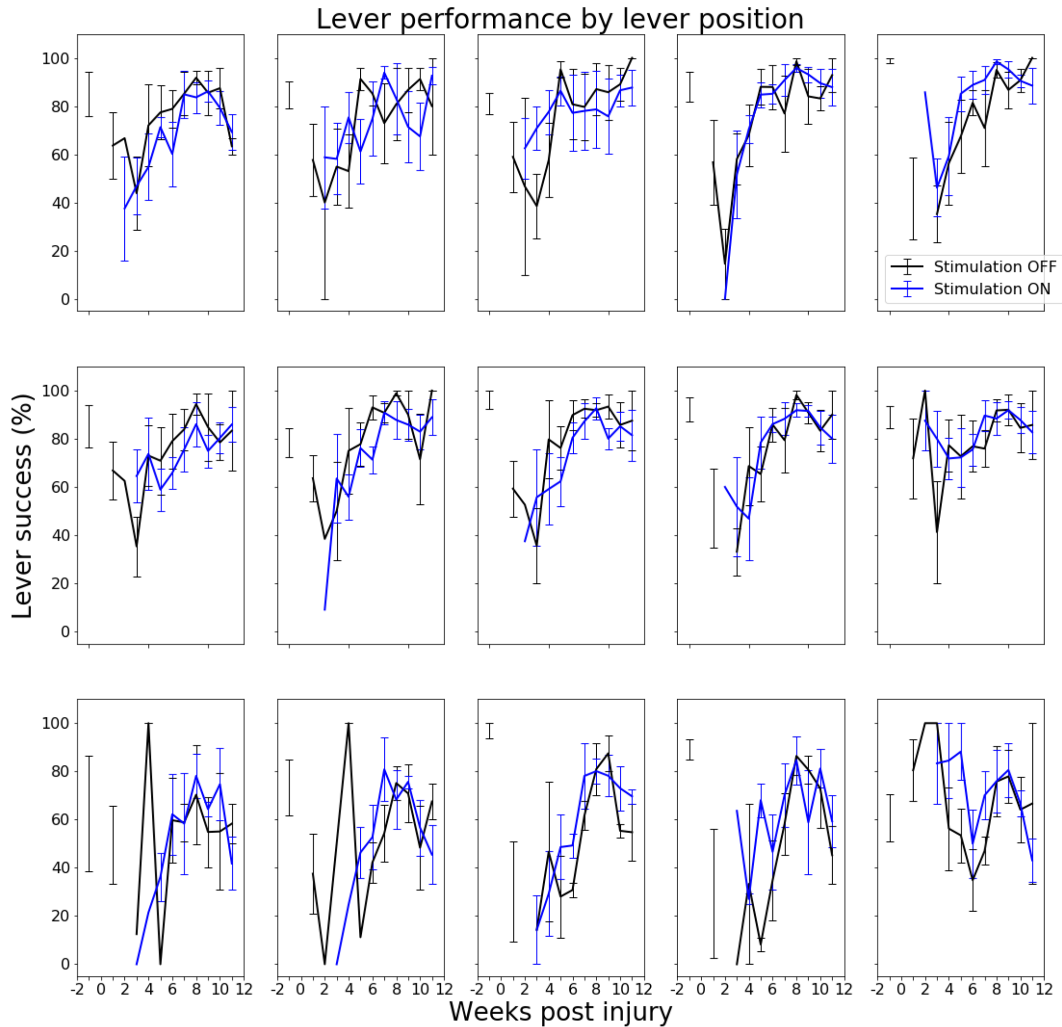


Figure 2.10. Weekly lever performance. Animals' overall performance on the lever task (top) and performance at each lever position (bottom) are plotted by week. Black lines = stimulation off, blue lines = stimulation on. Error bars = +/- standard error of the mean. Plots show the mean and S.E.M. of success rates across animals; individual animals' lever successes are defined as the percentage of successful lever presses achieved out of the total number of lever attempts at each position for each animal.

LUAT scores

We calculated separate LUAT scores for wall contacts and landings for each animal. A LUAT score of 0.5 reflects equal use of both limbs, while scores less than 0.5 reflect decreased use of the paralyzed limb as compared to the unaffected limb. Weekly scores reflect the

mean of the animals' individual scores for that week [Figure 2.11]. We calculated percent scores by dividing animals' scores by 0.5, to facilitate comparison with the percent scores used in their lever task performance evaluation and their IBB scores shown below.

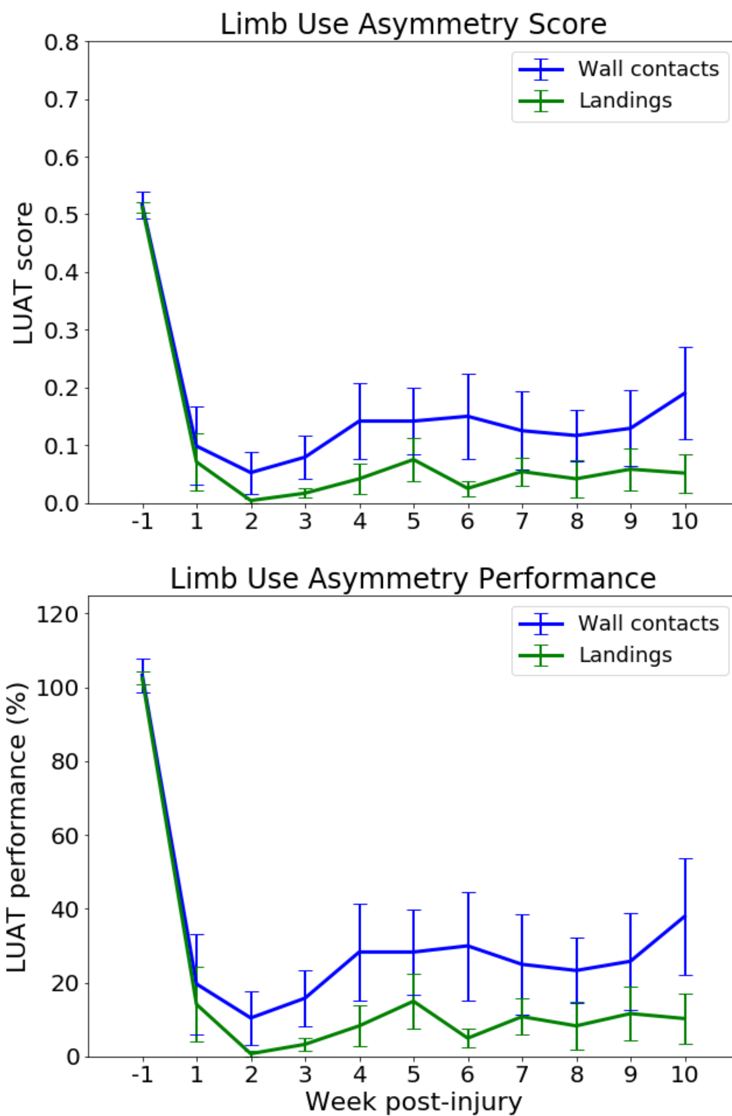


Figure 2.11. Limb Use Asymmetry Scores. Animals' mean LUAT scores (top) show the group weekly scores over time. Their percent performance (bottom) shows their scores as a percent of 0.5, which indicates equal limb use, to facilitate comparison with the lever performance (%) plots. Plots show the mean of individual animal scores +/- 1 standard deviation. Blue lines indicate wall contact scores, green lines indicate landing scores.

<u>IBB scores</u>

We calculated IBB scores for two cereal types for each animal: one spherical shape and one doughnut shape. Uninjured animals generally score 9.0, the maximum possible score, on both cereal types. Weekly scores shown here reflect the mean of the animals' individual scores for that week, where each individual score is the mean of three trials on that cereal shape [Figure 2.12]. We calculated percent scores by dividing animals' scores by 9.0 to facilitate comparison with the percent scores used in their lever task performance evaluation and the LUAT scores shown above.

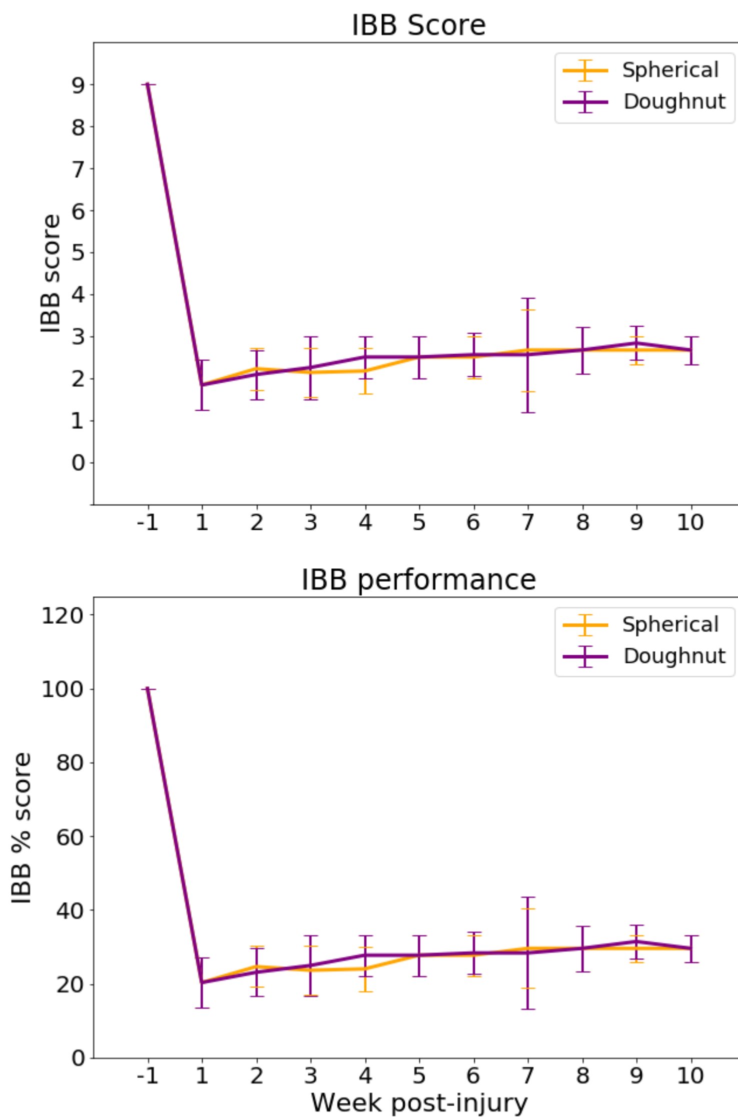


Figure 2.12. IBB Scores. Animals' mean IBB scores (top) show the group weekly scores over time. Their percent performance (bottom) shows their scores as a percent of 9.0, the maximum possible IBB score, to facilitate comparison with the lever performance (%) plots. Plots show the mean of individual animal scores +/- 1 standard deviation. Orange lines indicate scores for the spherical cereal shape, purple lines indicate scores for the doughnut cereal shape.

Statistics

Table 1 shows the fixed effects determined by the generalized linear mixed-effects regression model.

	Estimate	**Std. Error**	**Z value**	**Pr(>|z|)**
(Intercept)	0.41948	0.71989	0.583	0.5601
IBB (doughnut)	0.48459	0.07401	6.547	5.86e-11 ***
LUAT wall contacts	-0.06348	0.04957	-1.281	0.2003
LUAT landings	0.06225	0.03234	1.925	0.0542
Day	0.72529	0.03658	19.829	< 2e-16 ***
Stim. threshold	0.03293	0.04310	0.764	0.4449
Stim. status (on)	-0.09530	0.04103	-2.323	0.0202 *
Vertical lever pos.	0.58284	0.02205	26.432	< 2e-16 ***
Horizontal lever pos.	-0.12697	0.02021	-6.283	3.33e-10 ***

Significance codes: ******* 0.001, ****** 0.01, ***** 0.05

Table 1. Generalized linear mixed-effects regression results. The above table notes the fixed effects determined by the generalized linear mixed-effects regression model. The table shows the coefficient estimates (log-odds scale) and the standard error of these estimates. The values listed in column Pr(>|z|) indicate the significance of the effect, with asterisks (*****) denoting the significance level.

These results indicate that the day of training, horizontal lever position, vertical lever position, IBB score, and stimulation status have a significant effect on the prediction of the animals' scores at individual lever positions. The estimates indicate the size of the effect of each predictor variable on the prediction of lever success. The day variable has a positive

estimate, indicating a positive relationship between time and lever success. This reflects the trends in Figure 2.10, which show apparent increases in lever scores over time at many positions. The model results also reflect the pattern of lever success shown in the right panel of Figure 2.9, in which animals appear to perform better at rostral, proximal positions. The inverse relationship of the horizontal lever position and lever performance reflects this trend, as lower horizontal lever position values correspond to positions closer to the front of the arena, where animals exhibit greater success. The positive relationship between vertical lever position values and lever success also reflects the trend in Figure 2.9, as higher vertical lever position values correspond to lever positions closer to the platform. Additionally, there is a positive relationship between IBB scores and lever performance, and a negative relationship between the presence of stimulation and lever performance.

Discussion

This work presents a novel method for testing rat forelimb function that allows for the evaluation of animals with different injury severities. The automated lever pressing task is customizable for animals with different injury severities, and the mounting hardware allows testing of either limb as well as accommodating stimulation hardware (e.g. cables) through an open track in the lid. The rear chamber facilitates animal acclimation to the arena and provides a more open space for animal handling and cable attachment. The front chamber is narrower, promoting task focus and consistent positioning of the animal by limiting the space in which the animal might turn around. The system collects data automatically, requiring user intervention only to advance the lever for animals unable to reach a certain position.

The generally rising black lines in Figure 2.10 show that animals with incomplete unilateral cervical contusion injuries improve on the lever task over time. This demonstrates that the task can track animals' forelimb function recovery across multiple positions, enabling testing of groups that include animals with different injury severities. The arena may also function

as a physical rehabilitation system outside of intervention studies, as it prompts animals to explore lever positions beyond their capabilities and can track their progress over time.

The effects of the horizontal and vertical lever positions indicate that the animals generally perform better at positions closer to the front of the arena and closer to the platform (rostral, proximal positions). There seems to be a larger difference in the animals' performance between rows than between columns of positions. This may be a characteristic of their injuries, as it may be more difficult for animals to reach down in the vertical direction after spinal cord injury than it is for them to pull their forelimbs back in the horizontal direction. The horizontal movement appears to rely more on proximal limb function involving shoulder rotation, while reaching vertical positions requires more elbow extension, which is often impaired in this injury model. This difference may also reflect the rats' natural movements; it is possible that rats generally do not have to reach far in the vertical direction in their natural environments, but must execute shallow sifting behaviors when foraging for food.

The positive relationship between the IBB scores and lever performance indicates that animals with better distal limb function also perform better on the lever task. The IBB serves as a useful benchmark for new behavioral tasks, given its widespread use in rat studies; its utility in predicting lever success is encouraging. The IBB would serve as a useful complement to this lever task, as the two behavioral methods have their own unique advantages. The IBB meticulously evaluates distal forelimb and digit function during eating behavior, when the limb is positioned close to the animal's face, while the lever task evaluates the working range of motion of the limb when it positioned away from the face.

In this application, intraspinal microstimulation appears to impair animals' lever performance. This effect has many plausible explanations. First, we delivered stimulation immediately upon detection of the nosepoke; it is possible that some animals' limbs were not yet positioned to press the lever, and the stimulation disrupted their natural limb

positioning and timing. Our intent was to make the association of nosepoke and stimulation obvious to the animals, but future experiments should investigate whether adding a delay between the nosepoke and stimulation leads to improved performance with stimulation. It might also be useful to investigate the effects of different stimulation paradigms, such as sub-threshold stimulation. If the animals receive subthreshold stimulation continuously, then this stimulation might amplify any weak, descending motor signals that remain after injury, enabling limb movements only when these descending inputs activate their targets. This would allow for more natural control of limb movements, and may result in better lever press performance with stimulation than without.

Conclusion

We have developed a novel behavioral task for the evaluation of forelimb function that quantifies rats' ability to press a lever at each of fifteen individual lever positions covering a 15 cm^2 space within reach of the limb. This task and arena provide semi-automatic data collection before and after spinal cord injury and throughout the duration of treatment. The task and arena use liquid rewards, allowing animals with limited digit function and limited grasping abilities to receive rewards. The task is modifiable for either limb and can be used to evaluate many different treatment interventions, including treatments that require a physical cable or tether, such as electrical stimulation.

Supplementary materials

Using Shannon's approximation of McCreery's equation for maximum safe stimulation (78, 121):

$$\log\left(\frac{Q}{A}\right) = k - \log(Q)$$

Solve for *Q* (maximum safe charge):

$$\log\left(\frac{Q}{A}\right) = k - \log(Q)$$

$$\log\left(\frac{Q}{A}\right) + \log(Q) = k$$

$$\log\left(\frac{Q}{A} * Q\right) = k$$

$$\frac{Q^2}{A} = 10^k$$

$$Q^2 = 10^k * A$$

$$Q = \sqrt{10^k * A}$$

Convert charge *Q* (μC) to amplitude *Z* (μA), using *Q* μC / *t* seconds = *Z* μA:

$$Z = \frac{\sqrt{10^k * A}}{t}$$

Substituting *A* for the appropriate surface area formula for our wire electrode, the formula becomes:

$$Z = \frac{\sqrt{10^k * (\pi r^2 + \pi d h)}}{t}$$

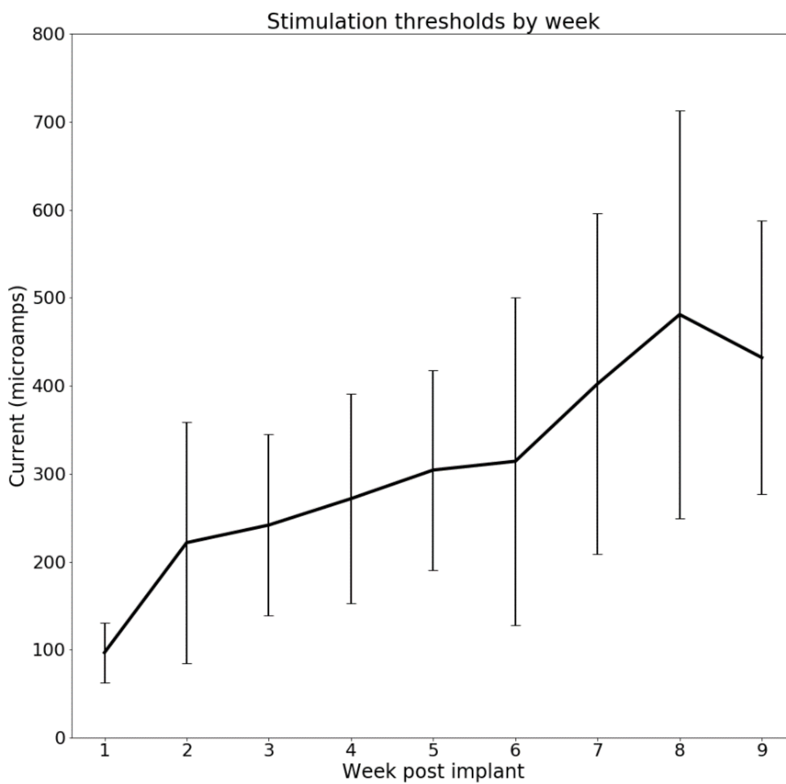
Where *r* is the radius of the wire, *d* is the diameter, and *h* is the length of exposed wire.

Using our pulse duration of 0.000300 seconds, wire diameter of 0.003048 cm, exposed wire length of 0.05 cm, and k=2.0, the maximum safe current becomes:

$$Z = \frac{\sqrt{10^2 * (\pi * (0.003048/2)^2 + (\pi * 0.003048 * 0.05))}}{0.000300}$$

$$Z = 734.9039 \cong 735\mu A$$

Supplementary Calculation 2.1. Determination of maximum safe stimulation current. We determined a maximum safe stimulation current based on Shannon's equation (121), where Q is the charge per phase (μC per phase), A is the surface area of the electrode (cm^2), and $1.5 < k < 2.0$ (78). We used k=2.0 for this calculation, and converted μC to μA to determine a maximum safe stimulation current given our electrode parameters (electrodes with diameter=30.48 μm, 0.5 mm exposed length, and pulse duration of 300 μsec).



Supplementary Figure 2.13. Stimulation thresholds. The mean currents required to produce an observable forelimb movement in the animals that successfully completed the study (n=6) are plotted by week. Line plot = mean of the movement threshold current on the stimulated channel for each animal. Error bars = +/- 1 standard deviation.

Chapter 3. Methods to rehabilitate forelimb function after spinal cord injury

3.0 Introduction

Electrical stimulation of the spinal cord attempts to treat one major effect of spinal cord injury by increasing the electrical activity within the spinal cord to induce or enable the activity necessary to activate paralyzed limbs. While electrical stimulation may promote lasting functional improvements to motor function (69, 73, 77), the optimal electrical stimulation parameters to restore long-term function remain unknown. In addition, electrical stimulation alone might not be sufficient to restore full function. It may be necessary to use other methods to completely restore motor function. Combining stimulation with approaches that aim to treat the other effects of spinal cord injury, such as impaired plasticity, tissue loss, and demyelination, might help restore function to paralyzed limbs. This chapter will discuss two such approaches – chondroitinase treatment and stem cell transplant – and the ways in which they might interact with electrical stimulation to enhance functional recovery.

One approach to restoring plasticity manipulates the extracellular matrix that limits synapse formation. Extracellular matrix components play an important role in guiding cell migration and axonal pathfinding during development (125). Chondroitin sulfate proteoglycans (CSPGs) are extracellular matrix proteins that form net-like structures around synapses toward the end of the critical period of development, restricting further reorganization and outgrowth (125).

Interestingly, the emergence of CSPGs requires activity during the critical period; inhibiting activity during this period reduces CSPG expression, but inhibiting activity during adulthood does not reduce their expression (66). Dissolving CSPGs in adulthood activates a period of experience-dependent plasticity of neural circuits (105, 106). This activity dependence makes CSPG manipulation a potentially effective approach to combine with electrical

stimulation. Dissolving CSPGs may open a period of plasticity during which electrical stimulation could help guide the formation of functional synapses. Spinal cord injury alone can trigger some plasticity, but the reorganization that occurs as a result can be undirected and maladaptive (9). For example, post-injury reorganization can strengthen circuits that promote spasticity (130). Electrical stimulation of specific circuits relevant to motor function may help to guide post-injury recovery toward the formation of helpful, rather than maladaptive, networks.

Dissolving CSPGs with the bacterial enzyme Chondroitinase ABC (ChABC) results in improved functional recovery after spinal cord injury (36, 37, 63, 126, 132), and promotes sprouting of descending motor projections (126) and increased conductance in fibers bypassing the lesion (55). As with electrical stimulation treatments, the recovery induced by dissolving CSPGs with ChABC treatment is not complete. Guiding ChABC-induced plasticity with direct stimulation of specific, functionally-relevant circuits (e.g. those necessary to execute a forelimb task) may enhance the recovery of motor function after spinal cord injury.

Spinal cord injury also results in cell damage and demyelination. Stem cell therapies designed to replace cells lost to injury might help to restore function, as these cells can differentiate into many different cell types. Transplanted stem and neural progenitor cells can differentiate into neurons and glia (90, 101, 133), increase myelination, promote axonal regeneration, and provide trophic support to the injured spinal cord (6). However, few cell transplantation therapies for spinal cord injury show more than modest benefits (6), and cell types must be carefully selected to avoid tumorigenesis (97, 134). Nonetheless, the potential benefits of cell therapies for the injured spinal cord merit further investigation.

Although functional recovery is modest, cell transplants do appear to improve motor function after spinal cord injury (24, 50), including improvements in forelimb functions (102, 115). Electrical stimulation may increase the functional benefits of cell therapies by

promoting cell survival (46), or by directing cells toward specific spinal cord sites. Direct current stimulation promotes stem cell migration in vitro (5, 85, 137) and acute electrical stimulation promotes transplanted neuron survival, host tissue myelination, and muscle re-innervation after peripheral nerve axotomy in vivo (46). These interactions support the investigation of a therapy that combines electrical stimulation with stem cell transplant.

This chapter presents our investigation of these approaches and their interactions with intraspinal electrical stimulation therapy. First, we explore the effect of combined intraspinal microstimulation and ChABC treatment in a rat model of spinal cord injury. Second, we explore the effect of stem cell therapy alone, as well as the effect of intraspinal stimulation on stem cell survival and differentiation.

3.1 Chondroitinase and electrical stimulation as a combined treatment for spinal cord injury

Methods

Subjects

We conducted experiments on 15 adult female Long Evans rats (>250 g). The University of Washington Institutional Animal Care and Use Committee approved all of our procedures.

Behavioral testing

We tested animals on the Limb Use Asymmetry Task (LUAT) (116), as described in Chapter 2. Briefly, this task scores the animals' use of the affected forelimb in a vertical cylinder exploration task, evaluating their ability to bear weight on the limb when contacting the cylinder and when landing from a reared position. We also tested animals on the Irvine, Beatties, and Bresnahan (IBB) cereal manipulation task (59), as described in Chapter 2. This task evaluates their distal forelimb function by scoring their ability to grasp and

manipulate two differently-shaped types of cereal. We tested animals on these tasks before injury, after injury, and for 8 weeks following spinal implant and ChABC treatment.

Spinal cord injury

All animals received a unilateral cervical spinal cord contusion injury as previously described (82). Briefly, we deeply anesthetized animals by injecting ketamine (80 mg/kg, intraperitoneal) and xylazine (12 mg/kg, intraperitoneal). Rats then received a lateralized contusion injury (0.7 mm displacement, 14 ms dwell time) at spinal segment C4 using a modified Ohio State injury device (81, 127). After their injury, our animals received buprenorphine (0.05 mg/kg, sub-cutaneous) for analgesia, once on the day of the injury and twice daily for two days thereafter.

Spinal implant and ChABC treatment

We divided our rats into three treatment groups. The first received chondroitinase and electrical stimulation (n=5). The second received chondroitinase and control spinal wires (n=5). The third received a vehicle-only injection and control wires.

Two weeks after spinal cord injury, we implanted each of our animals with an 8-10 channel platinum-iridium microwire stimulating array as described previously (69). We stripped approximately 0.3 mm of insulation from the wire tips to facilitate stimulation of a broader area and increase the likelihood of reaching the motor pools in the ventral grey matter. During the same surgery, we injected each animal with 3 μL of a solution containing a lentiviral vector expressing ChABC (LV-ChABC), described previously (13, 139), or with 3 μL of a control lentiviral construct expressing green fluorescent protein (LV-GFP).

Briefly, for this surgery we anesthetized our animals with isoflurane (1-3% in 100% oxygen, inhaled), exposed the injury site at C4, and performed hemilaminectomies at spinal segments C6-C7. We cut the dura and injected LV-ChABC or LV-GFP at a 1.5 mm depth at three sites: 1 μL immediately rostral to the injury site, 1 μL immediately rostral to the injury

site, and 1 μL at the site of the wire implant. We inserted wires 1.2-1.6 mm into the spinal cord at segments C6-C7 to target the forelimb motor pools. We then sutured the dura over the wires to secure them in place. We also positioned a reference wire in the surrounding muscle tissue. We secured the wire bundle again at T2 and routed it under the skin in a protective catheter to a connector affixed to the skull. Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) twice daily for analgesia.

<u>Spinal stimulation</u>

After one week of post-surgical recovery (three weeks after injury, one week after implant), we stimulated individual channels with single biphasic pulses (300 μsec per phase) to identify channels with forelimb movements. We increased currents at 10 μA intervals, starting at 10 μA until we saw a movement. We calculated a maximum safe stimulation current for our electrodes based on published guidelines (78, 121). Of the channels whose stimulation caused reliable forelimb movements, we chose the one with the lowest threshold for therapeutic stimulation. We stimulated our animals at the movement threshold current at frequencies described by a Gaussian distribution of 4 +/- 1.5 Hz biphasic, charge-balanced pulses in 15-minute blocks separated by 5-minute breaks as previously described (69). Stimulation sessions lasted 4-6 hours per day, occurring 5 days per week for 8 weeks.

<u>Allodynia testing</u>

One week prior to study completion, we tested the animals' mechanical sensitivity on all four paws using a simplified up-down method of the von Frey monofilament test (12), and recorded the force at which paw withdrawal occurred with a force meter attached to the monofilament.

<u>Anatomical tracing</u>

Two days prior to study completion, we injected 15 μL of wheat germ agglutinin (WGA) conjugated to an Alexa Fluor 555 fluorescent tag (Life Technologies Corp.) into the muscles

activated by each stimulation channel. For most animals, stimulation evoked elbow extension movements, and we inject WGA into the triceps muscles, dividing the volume across three injection sites.

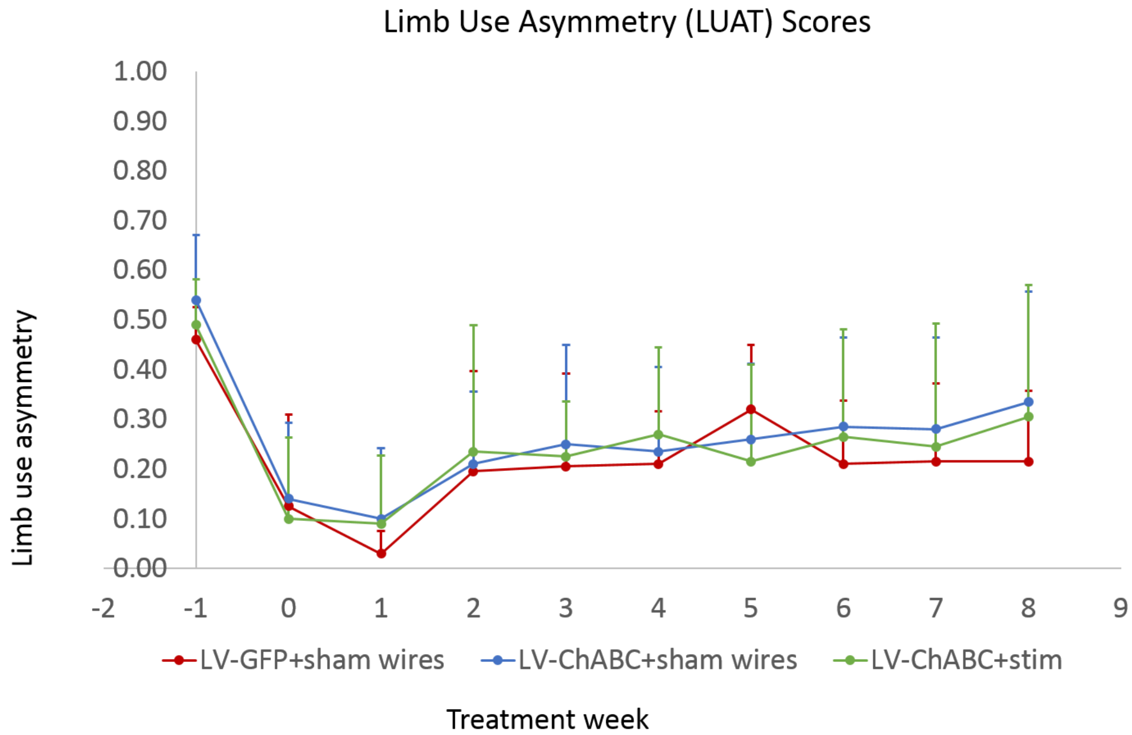
Tissue processing and histology

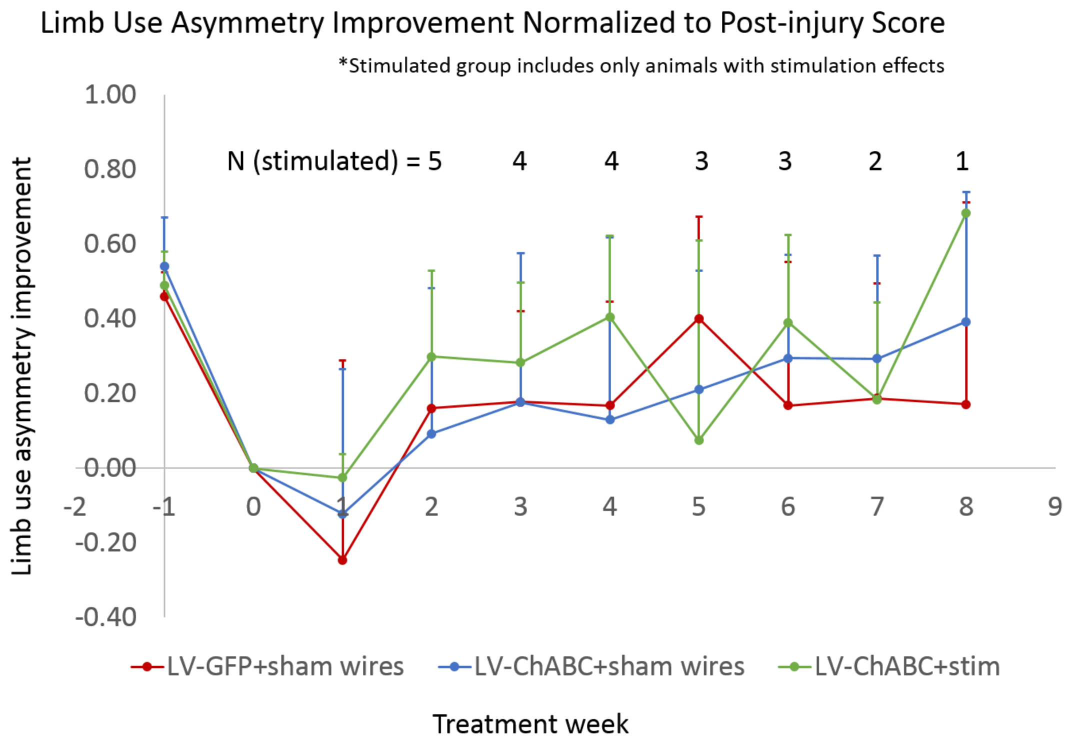
At the end of the study we deeply anesthetized animals with Beuthanasia-D Special (phenytoin/pentobarbital) and transcardially perfused them with 4% paraformaldehyde solution. We removed the spinal cord from the vertebral column, and cut the cervical cord into 2 mm blocks that we then embedded in Tissue-Tek Optimal Cutting Temperature Compound for freezing. We sectioned the frozen cervical cord blocks into 10 μm slices on a cryostat and mounted the sections on glass slides.

To evaluate how well our ChABC digested CSPGs, we immunostained sections with anti-chondroitin-4-sulfate antibody, which detects digested CSPG fragments. To visualize these immunostained fragments, we employed tyramide signal amplification using published protocols (8).

Results

There were no clear differences between the three treatment groups in the limb use asymmetry test [Figure 3.1]. We plotted the mean of each group's LUAT scores before injury (week -1), after injury (week 0), after implant (week 1), and for 7 weeks of stimulation (weeks 2-8). We also calculated the animals' improvement scores, as a ratio of the difference between their score in each week and their score at the first week of stimulation, over their baseline pre-injury score. Improvement scores do not show differences between groups, and the improvement scores of the stimulated group (green) appear to vary as animals begin to lose stimulation effects (weeks 5-8).





Limb use asymmetry improvement = (LUAT – wk 1 LUAT) / pre-injury LUAT

Figure 3.1. Limb Use Asymmetry Improvement. Limb use asymmetry scores for the three treatment groups across time (top) show no clear differences between groups. Error

bars = standard deviation of each group’s scores. Limb use asymmetry improvement, calculated as the difference between the animals’ weekly scores and their post-implant scores divided by their pre-injury scores (bottom panel), also shows no clear differences between groups. The bottom panel also shows the number of animals that retained observable stimulation effects as the study progressed. Error bars = standard deviation of each group’s improvement scores.

There were no clear differences between groups on the IBB task [Figure 3.2]. We plotted the mean of each group’s IBB scores before injury (week -1), after injury (week 0), after implant (week 1), and for 8 weeks following implant (weeks 2-9). We observed a decline in the number of animals with stimulation effects across the duration of the study, so we also plotted the performance of the two animals with the longest-lasting stimulation effects to compare their performance with the other two treatment groups [Figure 3.2, bottom panel]. Although the animals appear to perform better than the other two groups at the beginning of the study, their Week 0 scores are also greater than the means scores of the other two groups at week 0. The performance of these animals declines at week 6 and is inconsistent thereafter. To investigate whether this might be explained by changes in the animals’ stimulation thresholds, we plotted the movement threshold currents of the stimulated wires and the mean movement thresholds currents of the remaining (unstimulated) wires in each array [Figure 3.3]. Movement thresholds appear to increase at week 6 and become inconsistent thereafter, as the number of animals with stimulation effects decreases.

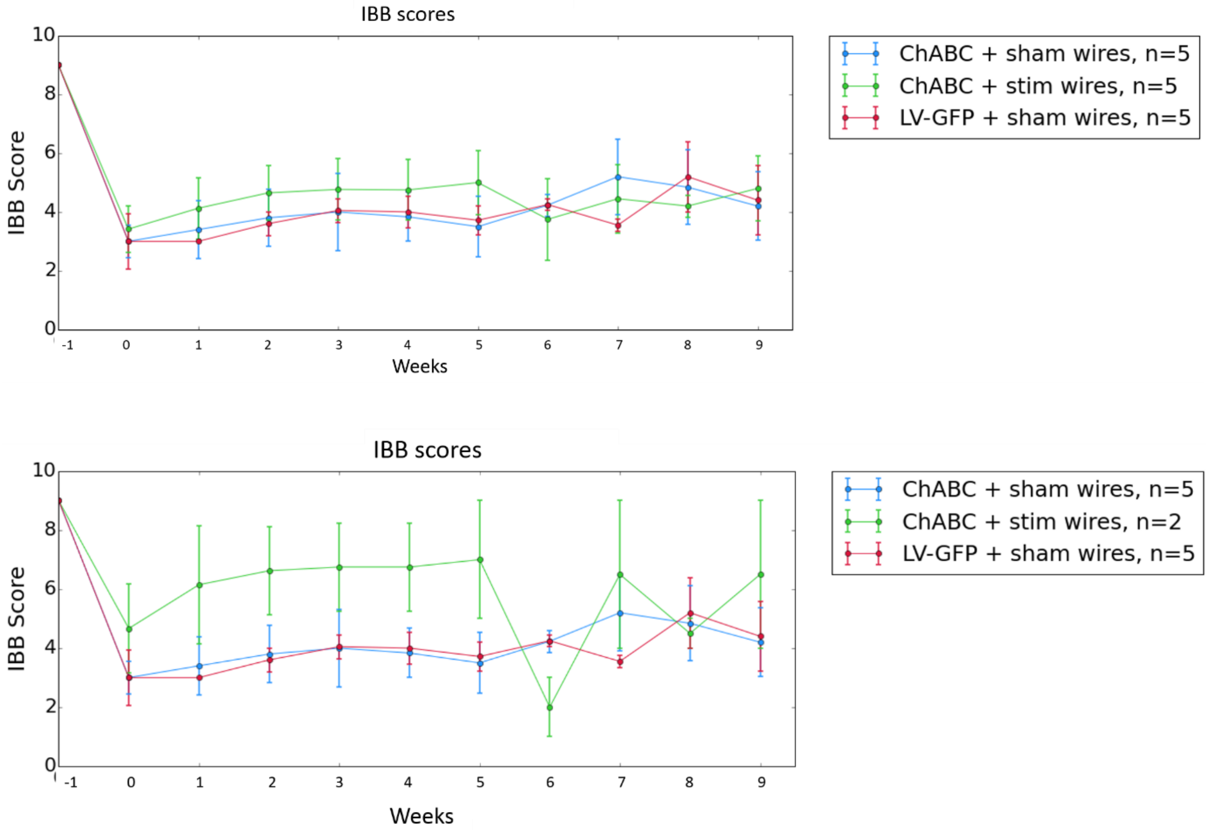


Figure 3.2. IBB scores. IBB scores for the three treatment groups across time (top) show no clear differences between groups. IBB scores of the two animals that retained stimulation effects for at least 7 weeks of the study (bottom), show a sharp decline at week 6 and inconsistent performance thereafter. Error bars = standard deviation of each group's weekly scores.

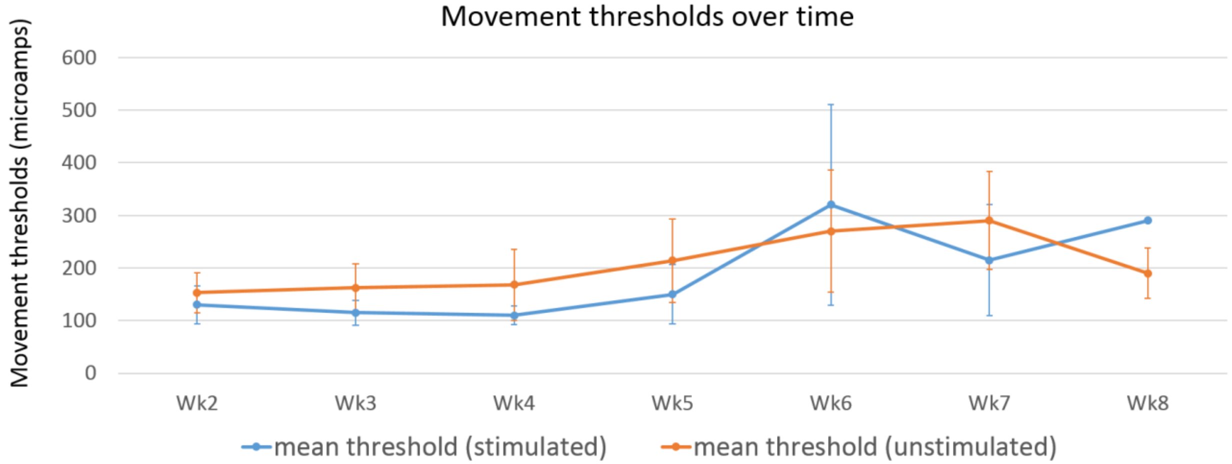


Figure 3.3. Movement thresholds of stimulated and unstimulated wires. The movement thresholds of the stimulated and unstimulated wires increase slowly over the 7

weeks of stimulation treatment. By week 5, only three animals remain with useable stimulation effects, and by week 8, only one animal has useable stimulation effects. Error bars = standard deviation of the movement threshold current for each group.

We also investigated whether there were any differences between groups on the Von Frey mechanical sensitivity test. The animals typically did not exhibit a withdrawal response or any indications of pain, allowing the user to push the probe up until it exerted enough force to push their paw up. Only then would they withdraw the limb. There were no significant differences between the groups in the force required to elicit withdrawal on any of the four limbs, using either the 15g probe or the blunt probe at the final week of the study (Week 8) (Student's t-test with Bonferroni correction, p>0.05) [Figure 3.4].

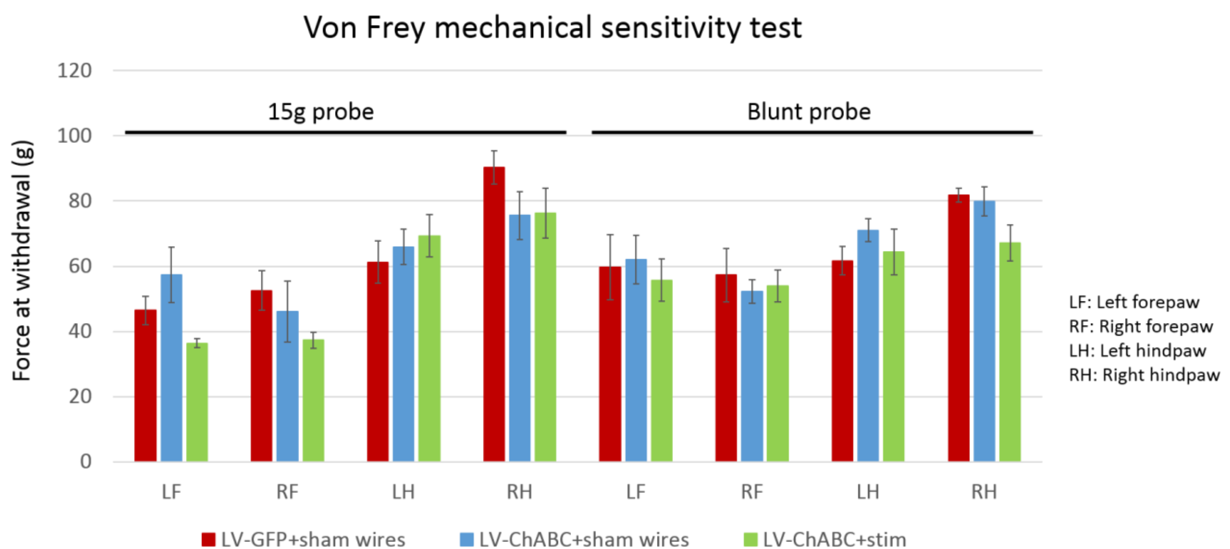


Figure 3.4. Mechanical force of probe at time of paw withdrawal. The bars show the mean mechanical force required to elicit the withdrawal of the left forepaw (LF), right forepaw (RF), left hindpaw (LH), and right hindpaw (RH) for each group. Error bars = standard error of the mean individual withdrawal force means for each group.

We also immunostained spinal cord tissue from the LV-ChABC treated animals to visualize CSPG digestion [Figure 3.5]. Digestion of CSPGs, as indicated by c-4-s immunolabeling, is inconsistent across LV-ChABC treated animals.

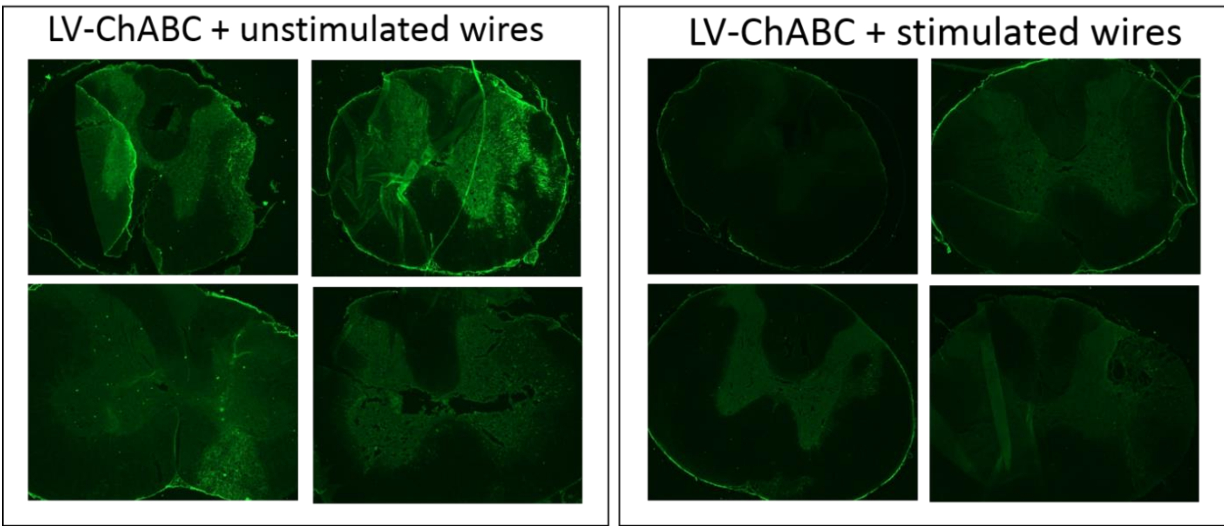


Figure 3.5. Examples of C-4-s expression. C-4-s labeling in animals that received lentiviral chondroitinase is inconsistent across animals. Figures show c-4-s immunoreactivity in the cervical spinal cord of eight LV-ChABC treated animals.

Discussion

There were no clear differences between the three treatment groups in recovery of forelimb function as measured by the IBB and LUAT tests, or in mechanical sensitivity or pain responses to the Von Frey probes. We speculate that inconsistent digestion of the CSPGs in our chondroitinase groups may contribute to this null result. Our early immunolabeling shows inconsistent CSPG digestion across the LV-ChABC treated animals [Figure 3.5]. Additionally, the amplification protocol used to evaluate CSPG digestion is not conducive to our slide mounted tissue preparation, as the multiple rinse steps often result in tissue lifting off from the slide and folding over or floating off the slide, despite our use of slides designed to improve tissue adhesion. Full assessment and quantification of CSPG digestion will likely require additional protocol modifications that improve tissue adhesion.

Even without CSPG digestion, one might expect to see a difference in forelimb function due to intraspinal microstimulation treatment. The loss of functional stimulation channels over time may explain the similarities between our stimulated and unstimulated LV-ChABC

groups. This loss may be explained by several factors, such as scar tissue buildup around the implants or movement of the wires in relation to the motor pools over time.

Alternatively, it is possible that intraspinal stimulation promotes functional recovery on specific, learned tasks, such as the forelimb pellet reaching task, and not on more general tasks, such as the LUAT. Previous intraspinal stimulation work indicates that this may indeed be the case (69). Similarly, chondroitinase treatment might be better suited to specific task rehabilitation than to general limb function rehabilitation (36). Additional studies investigating chondroitinase and intraspinal stimulation treatments should address both specific, trained tasks such as the forelimb reaching task and unskilled, general function tasks such as the IBB and LUAT to elucidate these differences.

3.2 Pluripotent stem cells and electrical stimulation as a combined treatment for spinal cord injury

This section contains two related experiments designed to investigate the effect of stem cell therapy alone and the effect of intraspinal stimulation on stem cell survival and differentiation. The first experiment (3.2.1) examines the effect of transplanted neural stem cells on the recovery of forelimb function after spinal cord injury. The second experiment (3.2.2) explores the effect of short-term therapeutic electrical stimulation on stem cell survival and differentiation in the spinal cord injury environment.

3.2.1 Neural stem cell treatment in a rat model of spinal cord injury

Methods

Cell culture

MRC5c3 human induced pluripotent neural stem cells and fibroblasts were cultured from previously generated stocks. Nutt et al. (29) describe the protocols for generating these

cells. Prior to transplant, we treated these cells with Accutase, pelleted and suspended in in 0.1 mg/ml DNase, 5 mM glucose solution at a 100,000 cells/μL concentration, as previously described (101).

Subjects

We conducted our experiments on stem cell treatments on 28 adult female Long Evans rats (>250 g). The University of Washington Institutional Animal Care and Use Committee approved all of our procedures.

Behavioral training and testing

We trained animals to proficiency on a skilled forelimb reaching task (FRT) (76) and tested them prior to spinal cord injury. We also tested the animals on the Limb Use Asymmetry Task (LUAT) (116), and the IBB cereal manipulation task (59), as described in Chapter 2. We tested animals on these tasks before injury, after injury, and for 14 weeks following spinal implant on the FRT and 15 weeks following implant on the LUAT and IBB.

Spinal cord injury

All animals received a unilateral cervical spinal cord contusion injury as described (82). Briefly, animals were deeply anaesthetized using injected ketamine (80 mg/kg, intraperitoneal) and xylazine (12 mg/kg, intraperitoneal), and then received a lateralized contusion injury (0.8 mm displacement, 14 ms dwell time) at spinal segment C4 using a modified Ohio State injury device (81, 127). Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) twice daily for analgesia.

Spinal implant and cell treatment

Animals were randomly assigned to three treatment groups, and received neural stem cells (n=9), fibroblasts (n=9), or sham injections of transplant solution without cells (n=10).

Four weeks after spinal cord injury, animals were anaesthetized using injected ketamine (80 mg/kg, intraperitoneal) and xylazine (12 mg/kg, intraperitoneal), and received cell or sham injections. One 1 μL injection was delivered at the rostral edge of the injury cavity and another 1 μL injection at the caudal edge of the injury cavity, each at a 60second-per-μL rate.

All animals received daily injections of cyclosporine (10 mg/kg, subcutaneous) following cell transplant to mitigate the immune reaction to transplanted cells.

Tissue processing and histology

At the culmination of the study, animals were deeply anesthetized with Beuthanasia-D Special (phenytoin/pentobarbital) and transcardially perfused with 4% paraformaldehyde solution. The spinal cord was removed from the spinal column, and the cervical cord was dissected and embedded longitudinally in Tissue-Tek Optimal Cutting Temperature Compound for freezing. Frozen tissue was sectioned into 20 μm slices on a cryostat and slide-mounted.

To identify cell transplants, sections were immunostained with a human nuclear marker (HuNu, Millipore). They were also immunostained with a standard astrocyte marker (GFAP, Millipore) and the DAPI nuclear counterstain.

Results

We did not observe any clear differences between the treatment groups on the skilled forelimb reaching task (FRT) [Figure 3.6]. The plotted FRT scores show the mean weekly scores for each group, beginning 5 weeks before cell transplant and continuing for 13 weeks after cell transplant. We normalized the scores to the animals' mean pre-injury scores (weeks -5, -4, and -3) by dividing the daily score for each animal by its mean pre-injury score and averaging across the week. Plots show the mean weekly normalized scores for the three treatment groups.

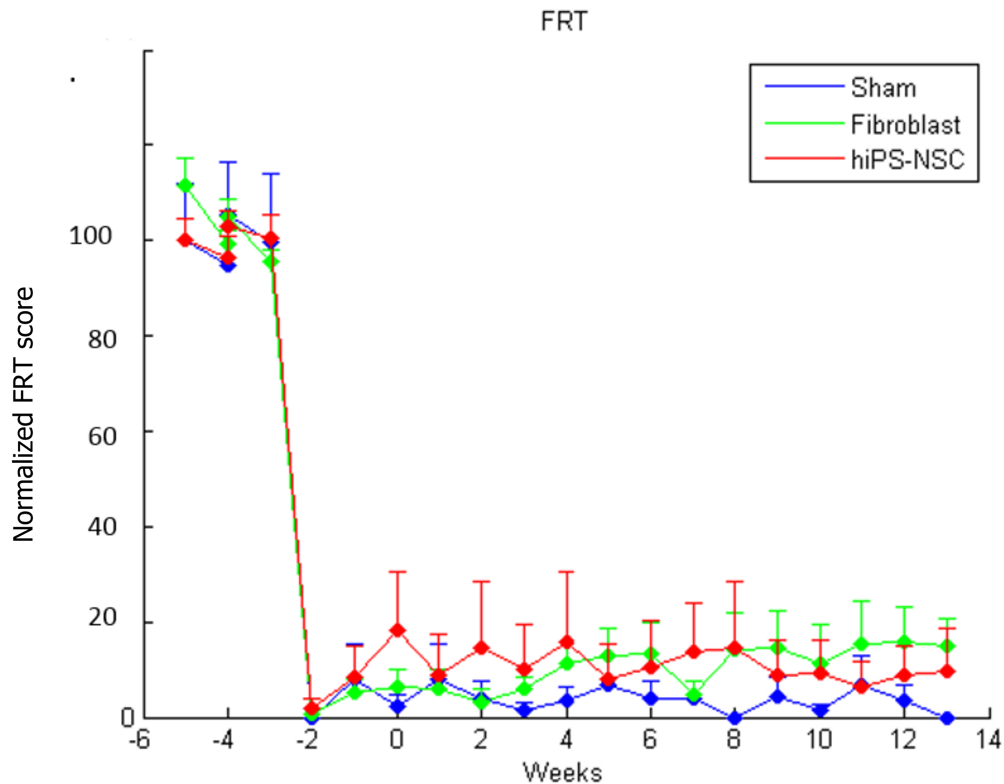


Figure 3.6. Forelimb reaching task scores. Plots show the mean weekly normalized FRT scores for the three treatment groups. Each weekly score is the mean of the animals' individual mean weekly scores. Error bars = +standard error of the mean weekly scores for each group.

We also did not observe any clear differences between groups on the forelimb asymmetry use test (LUAT) [Figure 3.7]. We also normalized the animals' scores to their pre-injury LUAT scores to facilitate comparison across groups and to avoid effects that might depend on individual animals' pre-injury limb-use preferences. As this is a weekly, rather than a daily test, these weekly scores are simply the mean of individual animals' weekly scores.

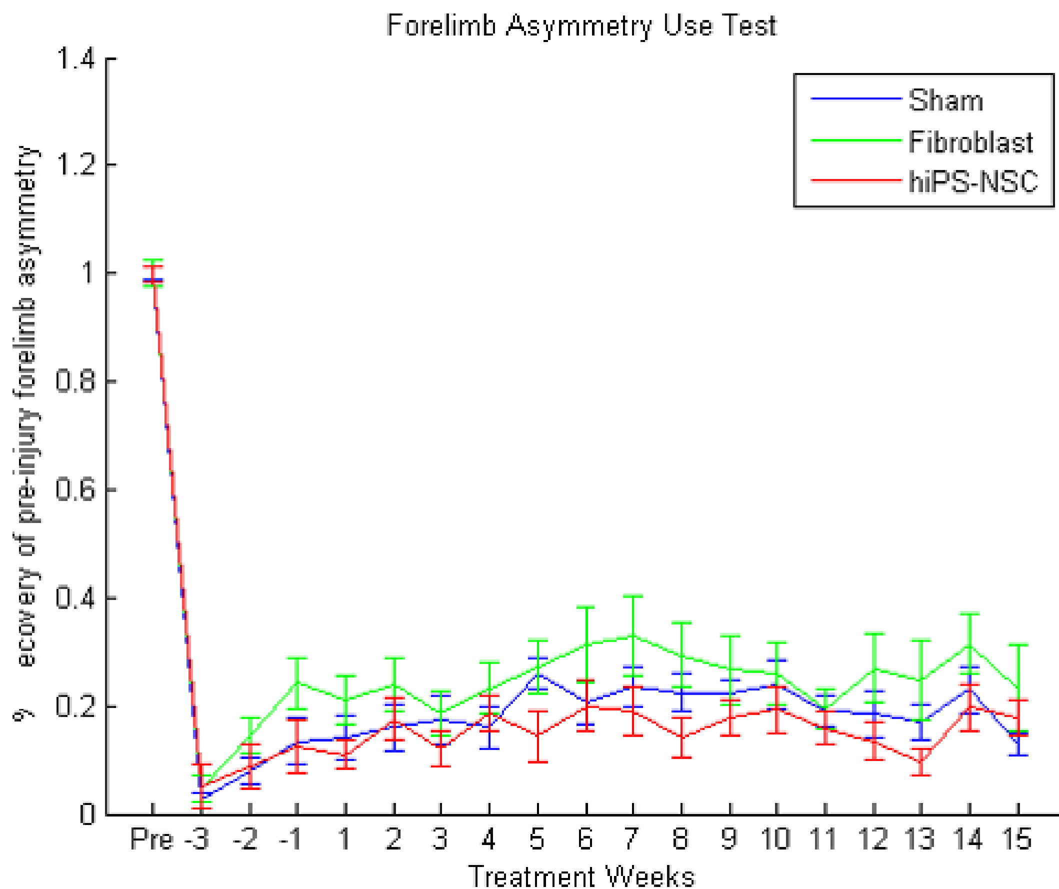


Figure 3.7. Forelimb asymmetry scores. Plots show the mean weekly normalized scores on the forelimb asymmetry use task. Error bars = +/- 1 standard deviation of the animals' weekly scores in each group.

We also did not observe any clear differences between groups on either IBB cereal type [Figure 3.8]. As this is also a weekly test, these weekly scores reflect the mean of individual animals' weekly scores.

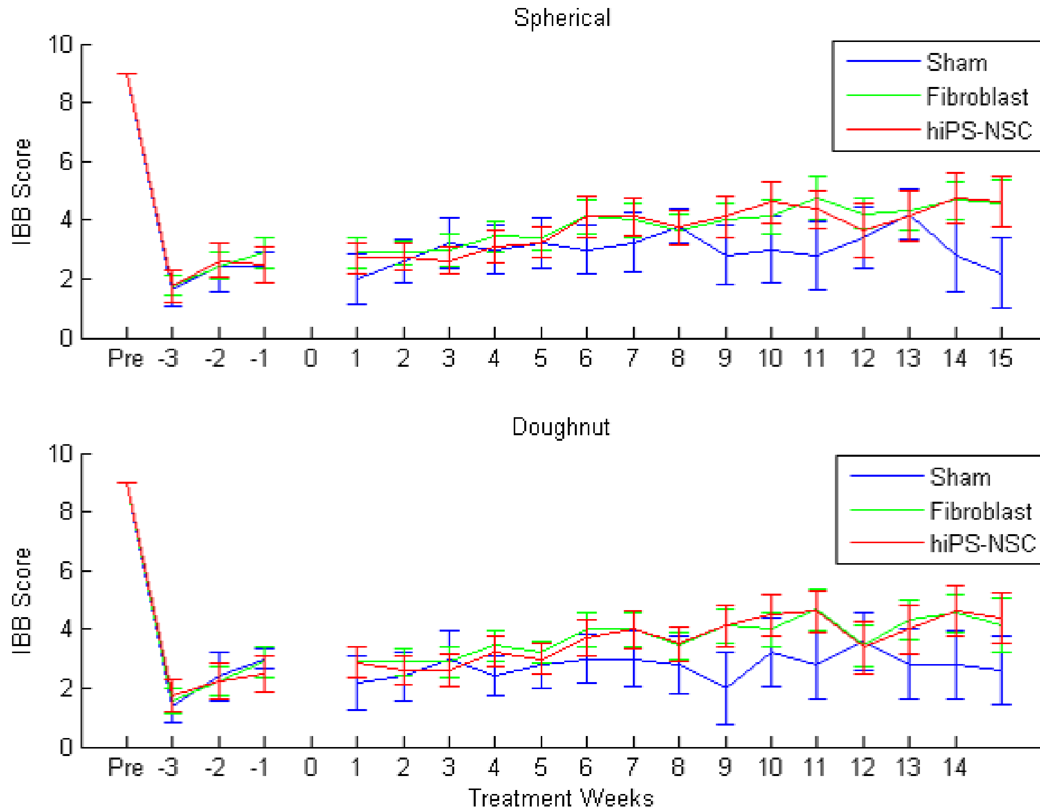


Figure 3.8. IBB scores. Plots show the mean weekly scores on the two cereal types evaluated in the IBB task. Error bars = +/- 1 standard deviation of the animals' weekly scores in each group.

We observed that the transplanted human neural stem cells we injected generally co-localized with astrocyte marker GFAP [Figure 3.9, left panel]. However, even in areas of high GFAP expression, not all transplanted cells co-localized with GFAP [Figure 3.9, right panel]. As the main goal of this study was to evaluate functional recovery with cell treatment, we did not quantify GFAP expression or further explore the fates of the transplanted cells.

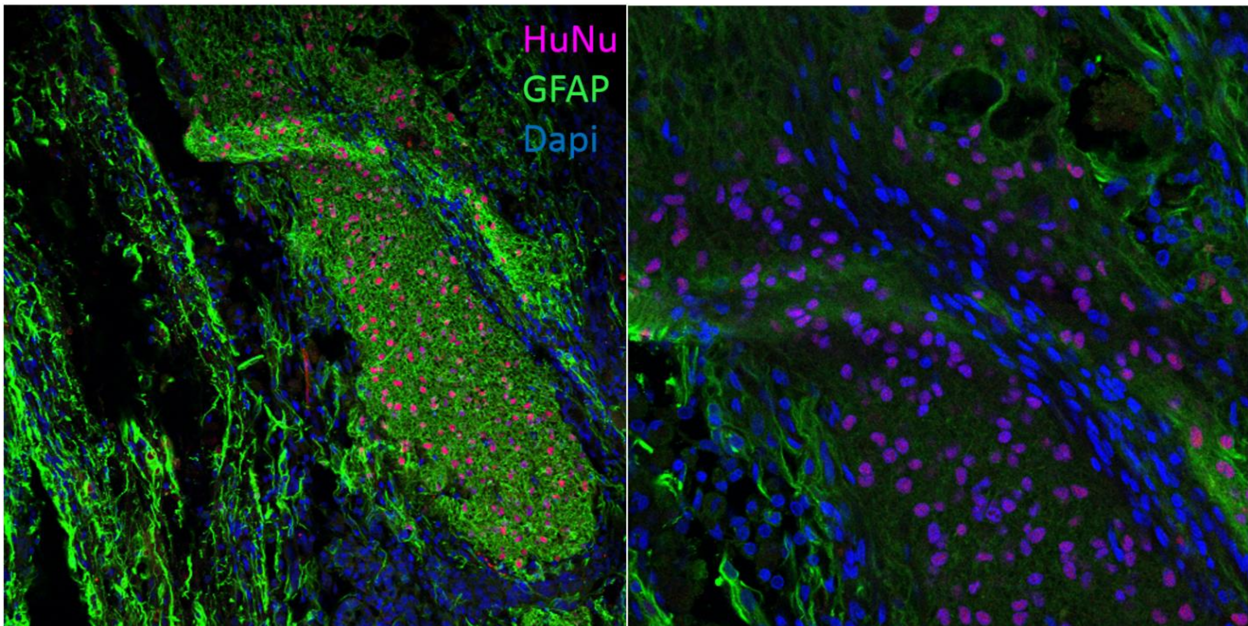


Figure 3.9. Transplanted cells in the injured spinal cord. Images show the expression of human nuclear marker HuNu and astrocyte marker GFAP with nuclear counterstain DAPI. The left panel shows transplanted cells (HuNu+) surrounded by GFAP. The right panel shows a small population of transplanted cells that are not co-localized with GFAP.

3.2.2 Combined electrical stimulation and neural stem cell treatment in a rat model of spinal cord injury

Methods

Cell culture

MRC5c3 human induced pluripotent neural stem cells and fibroblasts were cultured from previously generated stocks. Protocols for the generation of these cells are described elsewhere (101). Prior to injection, cells were treated with Accutase, pelleted and suspended in in 0.1mg/ml DNase, 5 mM glucose solution at a 100,000 cells/μL concentration, as previously described (101).

Subjects

Experiments were conducted on 16 adult female Long Evans rats (>250 g). The University of Washington Institutional Animal Care and Use Committee approved all of our procedures.

Spinal cord injury

All animals received a unilateral cervical spinal cord contusion injury. Briefly, we deeply anesthetized our animals by injecting ketamine (80 mg/kg, intraperitoneal) and xylazine (12 mg/kg, intraperitoneal). We then made a lateralized contusion injury (0.8 mm displacement, 14 ms dwell time) at spinal segment C4 using a modified Ohio State injury device (81, 127). Animals received buprenorphine (0.05 mg/kg, sub-cutaneous) twice daily for analgesia.

Spinal implant and cell treatment

We assigned animals randomly to three treatments groups. The first received neural stem cells and electrical intraspinal stimulation (n=6). The second received neural stem cells and unstimulated wire implants (n=5). The third received neural stem cells without wire implants (n=5).

Four weeks after spinal cord injury, we anesthetized the animals as before, and injected stem cells into their spinal cords. We made one 1 μL injection into the cord at the rostral edge of the injury cavity and another 1 μL injection at the caudal edge of the injury cavity. For both we injected at a rate of 1 μL/minute. After we made these cell injections, we implanted animals with 8-10 channel platinum-iridium microwire stimulating arrays as described previously (69).

All animals received daily injections of cyclosporine (10 mg/kg, subcutaneous) following cell transplant to mitigate the immune reaction to transplanted cells.

Spinal stimulation

After one week of post-surgical recovery, we stimulated through individual channels with single biphasic pulses (300 μsec per phase) to identify channels with forelimb movements. We increased currents at 10 μA intervals, starting at 10 μA until we saw a movement. We calculated a maximum safe stimulation current for our electrodes based on published guidelines (78, 121). Of the channels whose stimulation produced reliable forelimb movements, we chose the one with the lowest threshold for therapeutic stimulation. We stimulated our animals at half the movement threshold current, at frequencies described by a Gaussian distribution of 4 +/- 1.5 Hz biphasic, charge-balanced pulses in 15-minute blocks separated by 5-minute breaks as previously described (69). Stimulation sessions lasted 4 hours per day, occurring 5 days per week for 2 weeks.

Tissue processing and histology

We anesthetized animals deeply with Beuthanasia-D Special (phenytoin/pentobarbital) and transcardially perfused them with 4% paraformaldehyde solution. We removed the spinal cord from the vertebral column, and removed the cervical cord. We embedded the cervical cord longitudinally in Tissue-Tek Optimal Cutting Temperature Compound for freezing and cut 20 μm frozen sections on a cryostat. We mounted these sections on glass slides.

To evaluate how well stem cells survived, we immunostained sections with a human nuclear marker (HuNu, Millipore). To evaluate how well stem cells differentiated, we immunostained sections with a marker of Sox9, which is necessary to the maintenance of neural stem cells' multipotency in vitro and in vivo (118) and is involved in the specification of glial fates (67). We also stained cells with astrocyte marker GFAP (Millipore) and marked the nuclei of all neurons with the DAPI nuclear counterstain. We used unbiased stereological sampling methods with StereoInvestigator software (MBF Biosciences) to count cells and measure Sox9 expression.

Results

In all groups, few stem cells survived, with mean survival for the three treatment groups ranging from 4,000 – 8,000 cells [Figure 3.10, panel A]. On average, less than 25% of the surviving cells expressed multipotency marker Sox9 [Figure 3.10, panel B], indicating that the majority of surviving cells were no longer multipotent neural stem cells. The level of expression of Sox9 was highly variable from animal to animal. There were no significant differences between groups in the number of transplanted cells expressing Sox9 in each animal (Student's t-test, $p>0.05$). The majority of the transplanted cells co-localized with astrocyte marker GFAP [Figure 3.10, panel C], indicating that they may have matured to glial fates by the conclusion of the study.

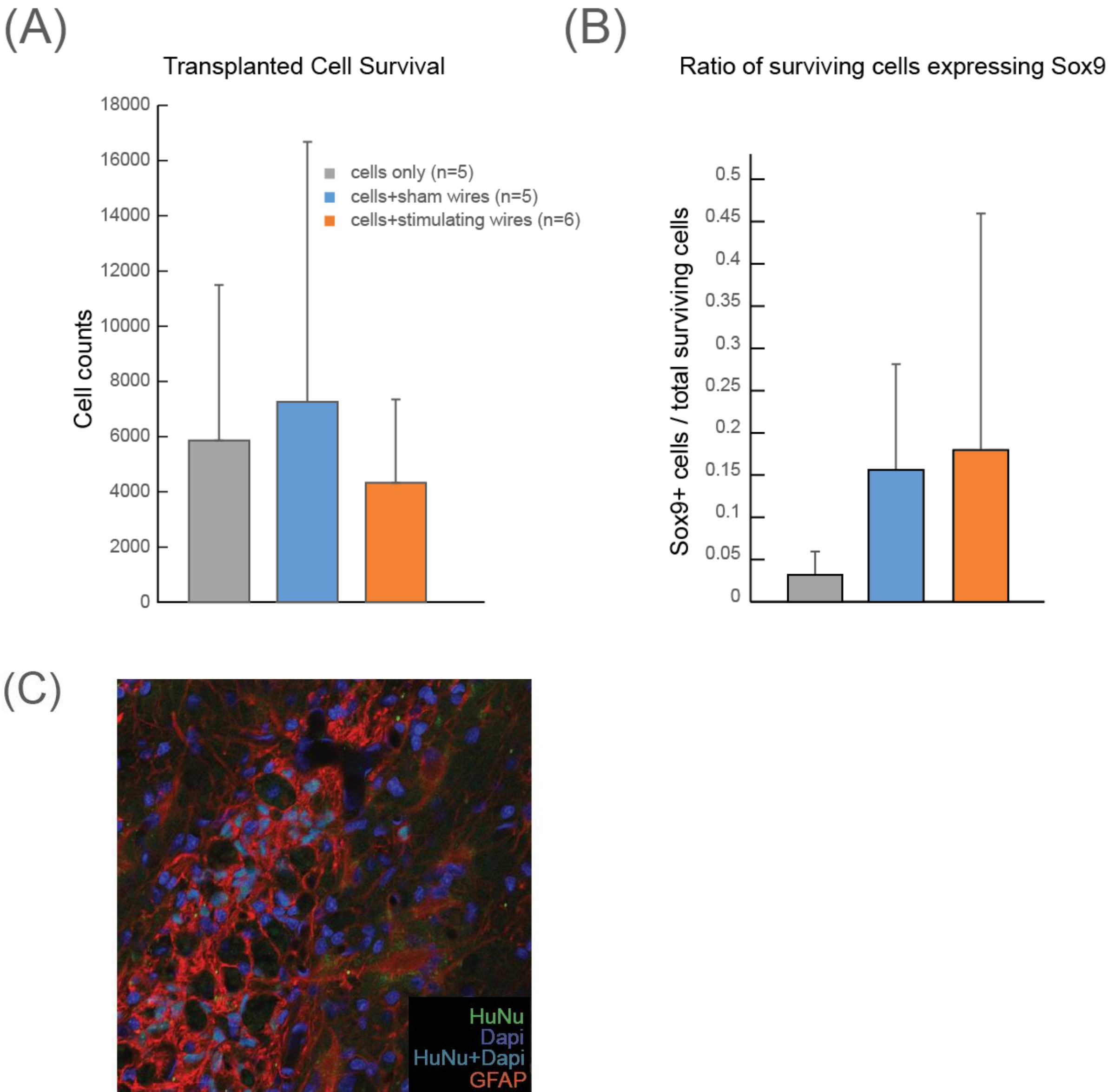


Figure 3.10. Effect of spinal implant and stimulation on cell survival and multipotency. We observed no significant differences in cell survival between the three groups (A) or in the expression of multipotency stem cell marker Sox9 (B) (Student's t-test, $p>0.05$). Bars indicate mean cell counts for each group. Error bars = + 1 standard deviation of the mean cell counts. Many transplanted cells also appeared to co-localize with astrocyte marker GFAP (C).

Our immunostaining also revealed that native cells clustered near the wire tips, with a visible piece of insulation in one of the cell clusters [Figure 3.11]. Cells also clustered near the wire tips even when stray pieces of insulation were not present [Figure 3.12], and appeared in stimulated as well as unstimulated animals [Figures 3.11 and 3.12].

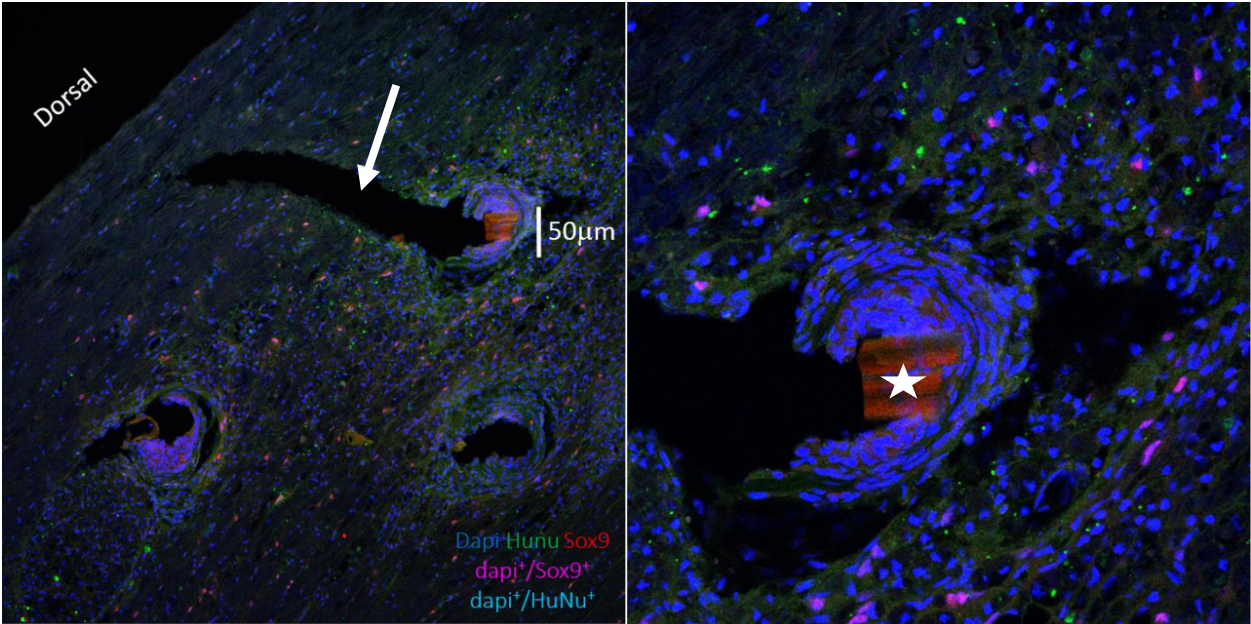


Figure 3.11. Wire tracks in spinal cord tissue. DAPI staining shows cells clustered around tips of wire tracks (wires explanted before staining). The white arrow in the left panel indicates an example wire track. A piece of insulation is still visible near tips of some wire tracks (white star, right panel). This animal's wires were briefly stimulated for weekly threshold testing, but were not chronically stimulated.

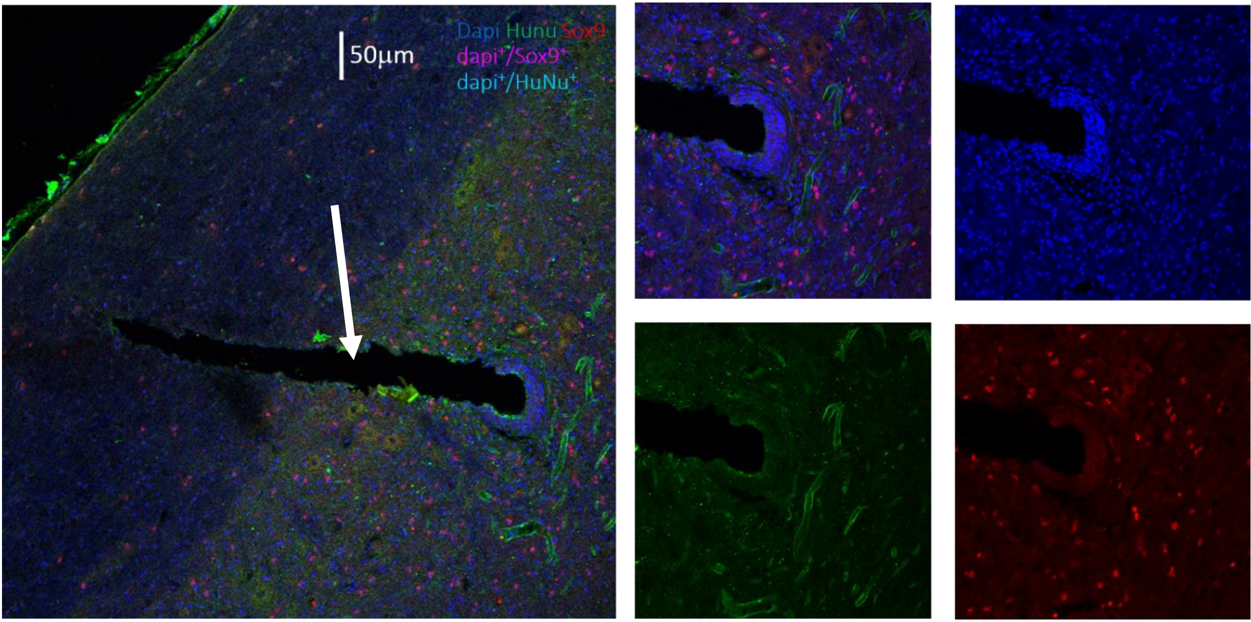


Figure 3.12. Wire tracks in spinal cord tissue. DAPI staining shows cells clustered around the tip of a wire track (wires explanted before staining). The white arrow indicates an example wire track. Some Sox9 expression is visible in the tissue surrounding the wire track (red inset panel), but the cells immediately adjacent to the wire tip location do not

express HuNu (green) or Sox9 (red). This animal received two weeks of chronic stimulation.

3.3 Discussion

These cell transplant studies did not demonstrate a benefit on the recovery of forelimb function of injecting neural stem cell or fibroblast transplants into the region of a spinal cord injury. Nor did they demonstrate any benefit of intraspinal microstimulation on transplanted neural stem cell survival or differentiation. In both studies, the majority of transplanted stem cells co-localized with astrocyte markers after transplant, indicating that they likely have differentiated toward glial fates. Prior work indicates, however, that at least in some cases, GFAP-positive cells with reduced Sox9 expression can still display multipotency (101). This is important to note, as undifferentiated cells can present a risk of overgrowth or tumor formation (134), especially in the injured spinal cord (72). Although there was no overgrowth evident in our animals, future investigations using this cell line would benefit from more in-depth analysis of transplanted cell fates to ensure safe and controlled transplanted stem cell survival and differentiation in the injured spinal cord.

The combined cell transplant and stimulation study showed that there may be an immune response to the electrodes that we implanted. Only two weeks after implant, an obvious cluster of cells had formed near the tips of the wires [Figures 3.11 and 3.12]. In some cases, pieces of wire insulation were visibly surrounded by cells [Figure 3.11], while in others, cells were cleanly arranged around the wire tips [Figure 3.12]. These cell clusters did not express the human nuclear marker of the transplanted cells, and they appeared on stimulated as well as unstimulated wires.

Reactive responses to nervous system implants are common (120), but may present challenges to long-term implant function. Cyclosporine, the immunosuppressant used to

mitigate the rats' immune reaction to the transplanted human cells, may increase the immune reaction to neural implants (120). Although previous studies using cyclosporine yielded better cell survival than this study (101), the reaction of cyclosporine with central nervous system implants merits further investigation. Other pharmacological approaches to mitigating the immune reaction to implants (e.g. dexamethasone) may improve implant longevity (120), and ought to be investigated in future studies seeking to investigate the effects of long-term intraspinal microstimulation.

It may also be useful to investigate the effects of different stimulation parameters (e.g. current amplitude, frequency, duration) on neural stem cell survival and differentiation in vitro to elucidate the stimulation and cell type combinations most likely to succeed in vivo. Some neural precursor cells migrate toward the cathode in the presence of a direct current electric field (1); it would be useful to test whether different types of stimulation, like pulsed biphasic stimulation of different pulse widths, affects migration. Although in vitro preparations cannot perfectly mimic the spinal cord injury microenvironment in a living animal system, this would allow for higher throughput screening and identification of potentially beneficial avenues for further in vivo investigation.

Chapter 4. Future Directions

4.1 Future Directions

Many open questions remain in regards to the reanimation and rehabilitation of limb function after spinal cord injury. The investigation of electrical stimulation, extracellular matrix manipulation, and stem cell transplant studies presented here investigates only the tip of the iceberg in spinal cord injury. Nonetheless, this work exemplifies the level of technical complexity characteristic of this research. Future studies ought to consider whether such complex approaches are necessary to realize a benefit to the spinal cord injury population, or whether simpler approaches might be more effective.

Chapter 2 presents an approach to the reanimation of paralyzed limbs that requires reliable recording and decoding of single-unit activity and subsequent encoding of that activity into a pattern of intraspinal microstimulation. Implanted stimulation arrays must reliably activate target motor units for this paradigm to successfully reanimate a paralyzed limb. The described paradigm, using one single unit to trigger stimulation at one spinal site, seems useful in reanimating a particular movement for a short period of time, but the results of this and the results described in subsequent chapters do not instill confidence in the hardware's potential to sustain function over months or years. Although stimulation of a single spinal site can evoke more complex movements than stimulation of an individual muscle, expanding this brain-triggered spinal stimulation paradigm to enable a full range of naturalistic hand and arm movements would still be challenging.

A simpler and more elegant approach might take advantage of the enabling effects of epidural and transcutaneous stimulation. Transcutaneous or epidural stimulation combined with a serotonergic agonist can enable volitional control of the paralyzed arm and hand and promote functional recovery (73). Increasing the baseline activity of the spinal cord can amplify descending cortical signals, eliminating the need for cortical signal decoding in

patients with incomplete spinal cord injuries. Even patients with clinically complete injuries retain some ability to modulate muscle activity (89); with enough such spared signals, and enough amplification of baseline spinal cord activity, one might eliminate the need for cortical decoding and thereby simplify the reanimation paradigm. These stimulation methods may also trigger a milder immune reaction (transcutaneous stimulation) or require simpler interventions to control the immune reaction. Local delivery of anti-inflammatory agents may be possible via an epidural catheter, limiting systemic side effects and supporting the longevity of electrical implants.

The lasting benefits of epidural and transcutaneous function merit further investigation. If such stimulation can reliably promote lasting changes after spinal cord injury, either alone or with serotonergic agonist treatment, additional approaches targeting the extracellular environment or cell transplant therapies may be unnecessary. If stimulation alone is not enough, combined treatment with plasticity-inducing agents such as chondroitinase may produce longer-lasting effects; however, chondroitinase treatment must be delivered in a controlled, consistent manner. Additional studies investigating the interaction of chondroitinase and electrical stimulation are necessary. If chondroitinase can enhance spike-timing plasticity, the two treatments might have an additive effect, but investigators should consider the possibility that broad epidural stimulation combined with chondroitinase treatment might also exacerbate the undirected, maladaptive plasticity present after spinal cord injury (9). It also seems logical that after sufficient post-injury rewiring, one might want to stop chondroitinase treatment to allow for consolidation of perineuronal nets around the newly strengthened synapses. An inducible viral construct could offer such control, and would also serve as an immensely useful research tool for a more detailed investigation of the mechanisms underlying the effects of chondroitinase treatment.

Stem cell transplant, although theoretically promising, requires substantial further investigation. The immune suppression required for rodent studies of human cell transplants

may be a barrier to investigating in rats the potential benefits of stem cells and combined treatments that involve electrical implants. Combined studies would also benefit from a detailed description of current spread. Before claiming that electrical stimulation has no effect on stem cell migration or differentiation, it would be clarifying to know that stem cells in a particular location were or were not within reach of the stimulation current. Other stimulation methods, such as optogenetics, might aid in stimulation-guided cell migration or differentiation by activating more specific sub-populations of cells.

Cell transplant studies might also benefit from simultaneous chondroitinase treatment. Chondroitinase promotes transplanted cell migration (57, 68) by limiting the inhibitory effects of the glial scar, which contains CSPGs. It might also enable the integration of transplanted cells into functional neural circuits by dissolving the CSPG chains in perineuronal nets surrounding stable synapses in the adult central nervous system. Such integration efforts may benefit from electrical stimulation paradigms that guide the transplanted cells toward functionally relevant networks.

All of these methods are at various stages of development, and are not likely to come together as a combined clinical treatment for many years, if at all. The investigation of their interactions remains an open problem for basic scientists to explore. Until then, epidural and transcutaneous stimulation methods likely provide the most immediate route to restoring function to paralyzed limbs.

References

1. **Ajiboye AB**, **Willett FR**, **Young DR**, **Memberg WD**, **Murphy BA**, **Miller JP**, **Walter BL**, **Sweet JA**, **Hoyen HA**, **Keith MW**, **Peckham PH**, **Simeral JD**, **Donoghue JP**, **Hochberg LR**, **Kirsch RF**. Restoration of reaching and grasping movements through brain-controlled muscle stimulation in a person with tetraplegia: a proof-of-concept demonstration. *Lancet* 389: 1821–1830, 2017.

2. **Alam M**, **Garcia-alias G**, **Shah PK**, **Gerasimenko Y**, **Roy RR**, **Edgerton VR**, **Angeles L**, **States U**, **Angeles L**, **States U**, **Angeles L**, **States U**, **Angeles L**, **States U**, **Brook S**. Evaluation of optimal electrode configurations for epidural spinal cord stimulation in cervical spinal cord injured rats. *J Neurosci Methods* 247: 50–57, 2016.

3. **Anderson KD**. Targeting recovery: priorities of the spinal cord-injured population. [Online]. *J Neurotrauma* 21: 1371–83, 2004. http://www.ncbi.nlm.nih.gov/pubmed/15672628.

4. **Angeli CA**, **Edgerton VR**, **Gerasimenko YP**, **Harkema SJ**. Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans. *Brain* 137: 1394–1409, 2014.

5. **Babona-Pilipos R**, **Droujinine IA**, **Popovic MR**, **Morshead CM**. Adult subependymal neural precursors, but not differentiated cells, undergo rapid cathodal migration in the presence of direct current electric fields. *PLoS One* 6: e23808, 2011.

6. **Badner A**, **Siddiqui AM**, **Fehlings MG**. Spinal cord injuries: How could cell therapy help? *Expert Opin Biol Ther* 0: 14712598.2017.1308481, 2017.

7. **Bamford JA**, **Mushahwar VK**. Intraspinal microstimulation for the recovery of function following spinal cord injury. *Prog Brain Res* 194: 227–239, 2011.

8. **Barritt A**, **Davies M**, **Marchand F**, **Hartley R**, **Grist J**, **Yip P**, **McMahon SB**, **Bradbury EJ**. Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. *J Neurosci* 26: 10856–67, 2006.

9. **Beauparlant J**, **van den Brand R**, **Barraud Q**, **Friedli L**, **Musienko P**, **Dietz V**, **Courtine G**. Undirected compensatory plasticity contributes to neuronal dysfunction after severe spinal cord injury. *Brain* 136: 3347–61, 2013.

10. **Bertelli JA**, **Mira JC**. Behavioral evaluating methods in the objective clinical assessment of motor function after experimental brachial plexus reconstruction in the rat. *J Neurosci Methods* 46: 203–208, 1993.

11. **Bi G**, **Poo M**. Synaptic Modification by Correlated Activity: Hebb's Postulate Revisited. *Annu Rev Neurosci* 24: 139–166, 2001.

12. **Bonin RP**, **Bories C**, **De Koninck Y**. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. *Mol Pain* 10: 26, 2014.

13. **Bosch KD**, **Bradbury EJ**, **Verhaagen J**, **Fawcett JW**, **McMahon SB**. Chondroitinase ABC promotes plasticity of spinal reflexes following peripheral nerve injury. *Exp Neurol* 238: 64–78, 2012.

14. **Bouton CE**, **Shaikhouni A**, **Annetta N V.**, **Bockbrader MA**, **Friedenberg DA**, **Nielson DM**, **Sharma G**, **Sederberg PB**, **Glenn BC**, **Mysiw WJ**, **Morgan AG**,

Deogaonkar M, **Rezai AR**. Restoring cortical control of functional movement in a human with quadriplegia. *Nature* 0: 1–13, 2016.

15. **Bradbury EJ**, **Moon LDF**, **Popat RJ**, **King VR**, **Bennett GS**, **Patel PN**, **Fawcett JW**, **McMahon SB**. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416: 636–40, 2002.

16. **van den Brand R**, **Heutschi J**, **Barraud Q**, **DiGiovanna J**, **Bartholdi K**, **Huerlimann M**, **Friedli L**, **Vollenweider I**, **Moraud EM**, **Duis S**, **Dominici N**, **Micera S**, **Musienko P**, **Courtine G**. Restoring voluntary control of locomotion after paralyzing spinal cord injury. *Science (80-)* 336: 1182–1185, 2012.

17. **van den Brand R**, **Heutschi J**, **Barraud Q**, **DiGiovanna J**, **Bartholdi K**, **Huerlimann M**, **Friedli L**, **Vollenweider I**, **Moraud EM**, **Duis S**, **Dominici N**, **Micera S**, **Musienko P**, **Courtine G**. Restoring voluntary control of locomotion after paralyzing spinal cord injury. *Science* 336: 1182–5, 2012.

18. **Campos RJ**, **Dimitrijevic MM**, **Faganel J**, **Sharkey PC**. Clinical evaluation of the effect of spinal cord stimulation on motor performance in patients with upper motor neuron lesions. *Appl Neurophysiol* 44: 141–151, 1981.

19. **Capogrosso M**, **Milekovic T**, **Borton D**, **Wagner F**, **Martin Moraud E**, **Mignardot J-B**, **Buse N**, **Gandar J**, **Barraud Q**, **Xing D**, **Rey E**, **Duis S**, **Jianzhong Y**, **Li WKDKQ**, **Detemple P**, **Denison T**, **Micera S**, **Bezard E**, **Bloch J**, **Courtine G**. A brain-spinal interface alleviating gait deficits after spinal cord injury in primates. *Nature* In Press: 284–288, 2016.

20. **Collinger JL**, **Wodlinger B**, **Downey JE**, **Wang W**, **Tyler-Kabara EC**, **Weber DJ**, **McMorland AJC**, **Velliste M**, **Boninger ML**, **Schwartz AB**. High-performance neuroprosthetic control by an individual with tetraplegia. *Lancet* 381: 557–64, 2013.

21. **Cook AW**, **Weinstein SP**. Chronic dorsal column stimulation in multiple sclerosis. Preliminary report. [Online]. *N Y State J Med* 73: 2868–72, 1973. http://www.ncbi.nlm.nih.gov/pubmed/4543587.

22. **Côté M-P**, **Murray M**, **Lemay MA**. Rehabilitation Strategies after Spinal Cord Injury: Inquiry into the mechanisms of success and failure. .

23. **Courtine G**, **Gerasimenko Y**, **Brand R Van Den**, **Yew A**, **Zhong H**, **Song B**, **Ao Y**, **Ichiyama RM**, **Lavrov I**, **Roy RR**, **Sofroniew M V**, **Edgerton VR**. Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. *Nat Neurosci* 12: 1333–1342, 2009.

24. **Cummings BJ**, **Uchida N**, **Tamaki SJ**, **Salazar DL**, **Hooshmand M**, **Summers R**, **Gage FH**, **Anderson AJ**. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci U S A* 102: 14069–74, 2005.

25. **Danner SM**, **Krenn M**, **Hofstoetter US**, **Toth A**, **Mayr W**, **Minassian K**. Body position influences which neural structures are recruited by lumbar transcutaneous spinal cord stimulation. *PLoS One* 11: 1–13, 2016.

26. **Devinsky O**. Electrical and magnetic stimulation of the central nervous system. Historical overview. [Online]. *Adv Neurol* 63: 1–16, 1993. papers3://publication/uuid/0C030802-DD77-4645-B13F-B0ACD4946EE2.

27. **Dimitrijevic MR**, **Illis LS**, **Nakajima K**, **Sharkey PC**, **Sherwood AM**. Spinal Cord

Stimulation for the Control of Spasticity in Patients with Chronic Spinal Cord Injury: II. Neurophysiologic Observations. *Cent Nerv Syst Trauma* 3: 129–143, 1986.

28. **Duru PO**, **Tillakaratne NJK**, **Kim JA**, **Zhong H**, **Stauber SM**, **Pham TT**, **Xiao MS**, **Edgerton VR**, **Roy RR**. Spinal neuronal activation during locomotor-like activity enabled by epidural stimulation and 5-HT agonists in spinal rats. *J Neurosci Res* 93: 1229–1239, 2015.

29. **Edgerton VR**, **Courtine G**, **Gerasimenko YP**, **Lavrov I**, **Ichiyama RM**, **Fong AJ**, **Cai LL**, **Otoshi CK**, **Tillakaratne NJK**, **Burdick JW**, **Roy RR**. Training Locomotor Networks. *Brain Res Rev* 57: 241–254, 2008.

30. **Fong AJ**, **Roy RR**, **Ichiyama RM**, **Lavrov I**, **Courtine G**, **Gerasimenko Y**, **Tai YC**, **Burdick J**, **Edgerton VR**. Recovery of control of posture and locomotion after a spinal cord injury : solutions staring us in the face. 175: 393–418, 2009.

31. **French JS**, **Anderson-Erisman KD**, **Sutter M**. What do spinal cord injury consumers want? A review of spinal cord injury consumer priorities and neuroprosthesis from the 2008 neural interfaces conference. *Neuromodulation* 13: 229–231, 2010.

32. **Gad P**, **Choe J**, **Shah P**, **Garcia-Alias G**, **Rath M**, **Gerasimenko Y**, **Zhong H**, **Roy RR**, **Edgerton V**. Sub-threshold spinal cord stimulation facilitates spontaneous motor activity in spinal rats. *J Neuroeng Rehabil* 10: 108, 2013.

33. **Gad P**, **Gerasimenko Y**, **Zdunowski S**, **Turner A**, **Sayenko D**, **Lu DC**, **Edgerton VR**. Weight Bearing Over-ground Stepping in an Exoskeleton with Non-invasive Spinal Cord Neuromodulation after Motor Complete Paraplegia. *Front Neurosci* 11: 1–8, 2017.

34. **Gad PN**, **Gerasimenko YP**, **Zdunowski S**, **Sayenko D**, **Haakana P**, **Turner A**, **Lu D**, **Roy RR**, **Edgerton VR**. Iron “ElectriRx” man: Overground stepping in an exoskeleton combined with noninvasive spinal cord stimulation after paralysis. *Proc Annu Int Conf IEEE Eng Med Biol Soc EMBS* 2015–Novem: 1124–1127, 2015.

35. **Gad PN**, **Roy RR**, **Zhong H**, **Lu DC**, **Gerasimenko YP**, **Edgerton VR**. Initiation of bladder voiding with epidural stimulation in paralyzed, step trained rats. *PLoS One* 9: 1–9, 2014.

36. **García-Alías G**, **Barkhuysen S**, **Buckle M**, **Fawcett JW**. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. *Nat Neurosci* 12: 1145–51, 2009.

37. **García-Alías G**, **Lin R**, **Akrimi SF**, **Story D**, **Bradbury EJ**, **Fawcett JW**. Therapeutic time window for the application of chondroitinase ABC after spinal cord injury. *Exp Neurol* 210: 331–338, 2008.

38. **Gerasimenko Y**, **Gorodnichev R**, **Moshonkina T**, **Sayenko D**, **Gad P**, **Reggie Edgerton V**. Transcutaneous electrical spinal-cord stimulation in humans. *Ann Phys Rehabil Med* 58: 225–231, 2015.

39. **Gerasimenko Y**, **Gorodnichev R**, **Puhov A**, **Moshonkina T**, **Savochin A**, **Selionov V**, **Roy RR**, **Lu DC**, **Edgerton VR**. Initiation and modulation of locomotor circuitry output with multisite transcutaneous electrical stimulation of the spinal cord in noninjured humans. *J Neurophysiol* 113: 834–842, 2015.

40. **Gerasimenko Y**, **Lu D**, **Modaber M**, **Zdunowski S**, **Gad P**, **Sayenko D**, **Morikawa**

E, **Haakana P**, **Ferguson AR**, **Roy RR**, **Edgerton VR**. Noninvasive Reactivation of Motor Descending Control after Paralysis. *J Neurotrauma* 32: 1968–1980, 2015.

41. **Gerasimenko Y**, **Roy RR**, **Edgerton VR**. Epidural Stimulation : Comparison of the Spinal Circuits That Generate and Control Locomotion in Rats, Cats and Humans. *Exp Neurol* 209: 417–425, 2008.

42. **Gerasimenko YP**, **Gad P**, **Sayenko DG**, **McKinney Z**, **Gorodnichev R**, **Puhov A**, **Moshonkina T**, **Savochin A**, **Selionov VA**, **Shigueva T**, **Tomilovskaya E**, **Kozlovskaya IB**, **Edgerton VR**. Integration of Sensory, Spinal, and Volitional Descending Inputs in Regulation of Human Locomotion. *J Neurophysiol* 116: 98–105, 2016.

43. **Gerasimenko YP**, **Ichiyama RM**, **Lavrov IA**, **Courtine G**, **Cai L**, **Zhong H**, **Roy RR**, **Edgerton VR**. Epidural Spinal Cord Stimulation Plus Quipazine Administration Enable Stepping in Complete Spinal Adult Rats. *J Neurophysiol* 98: 2525–2536, 2007.

44. **Giszter S.**, **Loeb E**, **Mussa-Ivaldi F.**, **Bizzi E**. Repeatable spatial maps of a few force and joint torque patterns elicited by microstimulation applied throughout the lumbar spinal cord of the spinal frog. *Hum Mov Sci* 19: 597–626, 2000.

45. **Grahn PJ**, **Lavrov IA**, **Sayenko DG**, **Van Straaten MG**, **Gill ML**, **Strommen JA**, **Calvert JS**, **Drubach DI**, **Beck LA**, **Linde MB**, **Thoreson AR**, **Lopez C**, **Mendez AA**, **Gad PN**, **Gerasimenko YP**, **Edgerton VR**, **Zhao KD**, **Lee KH**. Enabling Task-Specific Volitional Motor Functions via Spinal Cord Neuromodulation in a Human With Paraplegia. *Mayo Clin Proc* 92: 544–554, 2017.

46. **Grumbles RM**, **Liu Y**, **Thomas CM**, **Wood PM**, **Thomas CK**. Acute stimulation of transplanted neurons improves motoneuron survival, axon growth, and muscle reinnervation. *J Neurotrauma* 30: 1062–9, 2013.

47. **Harkema S**, **Gerasimenko Y**, **Hodes J**, **Burdick J**, **Angeli C**, **Chen Y**, **Ferreira C**, **Willhite A**, **Rejc E**, **Grossman RG**, **Edgerton VR**. Effect of Epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing, and assisted stepping after motor complete paraplegia: a case study. *Lancet* 377: 1938–1947, 2011.

48. **Harvey LA**, **Glinsky J V**, **Bowden JL**. The effectiveness of 22 commonly administered physiotherapy interventions for people with spinal cord injury: a systematic review. *Spinal Cord* 47: 184–195, 2016.

49. **Hochberg LR**, **Bacher D**, **Jarosiewicz B**, **Masse NY**, **Simeral JD**, **Vogel J**, **Haddadin S**, **Liu J**, **Cash SS**, **van der Smagt P**, **Donoghue JP**. Reach and grasp by people with tetraplegia using a neurally controlled robotic arm. *Nature* 485: 372–375, 2013.

50. **Hofstetter CP**, **Holmström N a V**, **Lilja J a**, **Schweinhardt P**, **Hao J**, **Spenger C**, **Wiesenfeld-Hallin Z**, **Kurpad SN**, **Frisén J**, **Olson L**. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nat Neurosci* 8: 346–53, 2005.

51. **Hofstoetter US**, **Krenn M**, **Danner SM**, **Hofer C**, **Kern H**, **Mckay WB**, **Mayr W**, **Minassian K**. Augmentation of Voluntary Locomotor Activity by Transcutaneous Spinal Cord Stimulation in Motor-Incomplete Spinal Cord-Injured Individuals. *Artif Organs* 39: E176–E186, 2015.

52. **Hofstoetter US**, **McKay WB**, **Tansey KE**, **Mayr W**, **Kern H**, **Minassian K**. Modification of spasticity by transcutaneous spinal cord stimulation in individuals with

incomplete spinal cord injury. *J Spinal Cord Med* 37: 202–11, 2014.

53. **Holinski BJ**, **Everaert DG**, **Mushahwar VK**, **Stein RB**. Real-time control of walking using recordings from dorsal root ganglia. *J Neural Eng* 10: 56008, 2013.

54. **Holinski BJ**, **Mazurek KA**, **Everaert DG**, **Toossi A**, **Lucas-Osma AM**, **Troyk P**, **Etienne-Cummings R**, **Stein RB**, **Mushahwar VK**. Intraspinal microstimulation produces over-ground walking in anesthetized cats. *J Neural Eng* 13: 56016, 2016.

55. **Hunanyan AS**, **García-Alías G**, **Alessi V**, **Levine JM**, **Fawcett JW**, **Mendell LM**, **Arvanian VL**. Role of chondroitin sulfate proteoglycans in axonal conduction in Mammalian spinal cord. *J Neurosci* 30: 7761–9, 2010.

56. **Ichiyama RM**, **Gerasimenko YP**, **Zhong H**, **Roy RR**, **Edgerton VR**. Hindlimb stepping movements in complete spinal rats induced by epidural spinal cord stimulation. *Neurosci Lett* 383: 339–344, 2005.

57. **Ikegami T**, **Nakamura M**, **Yamane J**, **Katoh H**, **Okada S**, **Iwanami A**, **Watanabe K**, **Ishii K**, **Kato F**, **Fujita H**, **Takahashi T**, **Okano HJ**, **Toyama Y**, **Okano H**. Chondroitinase ABC combined with neural stem/progenitor cell transplantation enhances graft cell migration and outgrowth of growth-associated protein-43-positive fibers after rat spinal cord injury. *Eur J Neurosci* 22: 3036–46, 2005.

58. **Irvine K-A**, **Ferguson AR**, **Mitchell KD**, **Beattie SB**, **Beattie MS**, **Bresnahan JC**. A novel method for assessing proximal and distal forelimb function in the rat: the Irvine, Beatties and Bresnahan (IBB) forelimb scale. *J Vis Exp* : 1–9, 2010.

59. **Irvine KA**, **Ferguson AR**, **Mitchell KD**, **Beattie SB**, **Lin A**, **Stuck ED**, **Huie JR**, **Nielson JL**, **Talbott JF**, **Inoue T**, **Beattie MS**, **Bresnahan JC**. The Irvine, Beatties, and Bresnahan (IBB) forelimb recovery scale: An assessment of reliability and validity. *Front Neurol* 5 JUL: 1–19, 2014.

60. **Iwahara T**, **Atsuta Y**, **Garcia-Rill E**, **Skinner RD**. Spinal cord stimulation-induced locomotion in the adult cat. *Brain Res Bull* 28: 99–105, 1992.

61. **Jackson A**, **Mavoori J**, **Fetz EE**. Long-term motor cortex plasticity induced by an electronic neural implant. *Nature* 444: 56–60, 2006.

62. **James ND**, **Shea J**, **Muir EM**, **Verhaagen J**, **Schneider BL**, **Bradbury EJ**. Chondroitinase gene therapy improves upper limb function following cervical contusion injury. *Exp Neurol* 271: 131–135, 2015.

63. **Jefferson SC**, **Tester NJ**, **Howland DR**. Chondroitinase ABC promotes recovery of adaptive limb movements and enhances axonal growth caudal to a spinal hemisection. *J Neurosci* 31: 5710–20, 2011.

64. **Jilge B**, **Minassian K**, **Rattay F**, **Pinter MM**, **Gerstenbrand F**, **Binder H**, **Dimitrijevic MR**. Initiating extension of the lower limbs in subjects with complete spinal cord injury by epidural lumbar cord stimulation. *Exp Brain Res* 154: 308–326, 2004.

65. **Jung R**, **Belanger A**, **Kanchiku T**, **Fairchild M**, **Abbas JJ**. Neuromuscular stimulation therapy after incomplete spinal cord injury promotes recovery of interlimb coordination during locomotion. *J Neural Eng* 6: 55010, 2009.

66. **Kalb RG**, **Hockfield S**. Electrical activity in the neuromuscular unit can influence the molecular development of motor neurons. *Dev Biol* 162: 539–548, 1994.

67. **Kang P**, **Lee HK**, **Glasgow SM**, **Finley M**, **Donti T**, **Gaber ZB**, **Graham BH**, **Foster AE**, **Novitch BG**, **Gronostajski RM**, **Deneen B**. Sox9 and NFIA coordinate a transcriptional regulatory cascade during the initiation of gliogenesis. *Neuron* 74: 79–94, 2012.

68. **Karimi-Abdolrezaee S**, **Eftekharpour E**, **Wang J**, **Schut D**, **Fehlings MG**. Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord. *J Neurosci* 30: 1657–76, 2010.

69. **Kasten MR**, **Sunshine MD**, **Secrist ES**, **Horner PJ**, **Moritz CT**. Therapeutic intraspinal microstimulation improves forelimb function after cervical contusion injury. *J Neural Eng* 10: 44001, 2013.

70. **Krause P**, **Edrich T**, **Straube A**. Lumbar repetitive magnetic stimulation reduces spastic tone increase of the lower limbs. *Spinal Cord* 42: 67–72, 2004.

71. **Lemay M a**, **Grill WM**. Modularity of motor output evoked by intraspinal microstimulation in cats. *J Neurophysiol* 91: 502–14, 2004.

72. **Lopez-Serrano C**, **Torres-Espin A**, **Hernandex J**, **Alvarez-Palomo AB**, **Requena J**, **Gasull X**, **Edel MJ**, **Navarro X**. Effects of the spinal cord injury environment on the differentiation capacity of human neural stem cells derived from induced pluripotent stem cells. *Cell Transplant* 25: 1–37, 2016.

73. **Lu DC**, **Edgerton VR**, **Modaber M**, **AuYong N**, **Morikawa E**, **Zdunowski S**, **Sarino ME**, **Sarrafzadeh M**, **Nuwer MR**, **Roy RR**, **Gerasimenko Y**. Engaging cervical spinal cord networks to reenable volitional control of hand function in tetraplegic patients. *Neurorehabil Neural Repair* 30: 951–962, 2016.

74. **Mazurek KA**, **Holinski BJ**, **Everaert DG**, **Stein RB**, **Etienne-Cummings R**, **Mushahwar VK**. Feed forward and feedback control for over-ground locomotion in anaesthetized cats. *J Neural Eng* 9: 26003, 2012.

75. **McKenna JE**, **Prusky GT**, **Whishaw IQ**. Cervical motoneuron topography reflects the proximodistal organization of muscles and movements of the rat forelimb: a retrograde carbocyanine dye analysis. [Online]. *J Comp Neurol* 419: 286–96, 2000. http://www.ncbi.nlm.nih.gov/pubmed/10723005.

76. **McKenna JE**, **Whishaw IQ**. Complete compensation in skilled reaching success with associated impairments in limb synergies, after dorsal column lesion in the rat. *J Neurosci* 19: 1885–1894, 1999.

77. **McPherson JG**, **Miller RR**, **Perlmutter SI**. Targeted, activity-dependent spinal stimulation produces long-lasting motor recovery in chronic cervical spinal cord injury. *PNAS* 112: 12193–12198, 2015.

78. **Merrill DR**, **Bikson M**, **Jefferys JGR**. Electrical stimulation of excitable tissue: Design of efficacious and safe protocols. *J Neurosci Methods* 141: 171–198, 2005.

79. **Metz G a**, **Whishaw IQ**. The ladder rung walking task: a scoring system and its practical application. *J Vis Exp* : 2–5, 2009.

80. **Minassian K**, **Hofstoetter US**, **Danner SM**, **Mayr W**, **Bruce JA**, **McKay WB**, **Tansey KE**. Spinal Rhythm Generation by Step-Induced Feedback and Transcutaneous Posterior Root Stimulation in Complete Spinal Cord-Injured Individuals. *Neurorehabil Neural Repair* 30: 233–43, 2016.

81. **Mondello SE**, **Kasten MR**, **Horner PJ**, **Moritz CT**. Therapeutic intraspinal stimulation to generate activity and promote long-term recovery. *Front Neurosci* 8: 1–7, 2014.

82. **Mondello SE**, **Sunshine MD**, **Fischedick AE**, **Moritz CT**, **Horner PJ**. A cervical hemi-contusion spinal cord injury model for the investigation of novel therapeutics targeting proximal and distal forelimb functional recovery. *J Neurotrauma* 32: 1994–2007, 2015.

83. **Montoya CP**, **Campbell-Hope LJ**, **Pemberton KD**, **Dunnett SB**. The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. *J Neurosci Methods* 36: 219–228, 1991.

84. **Moraud EM**, **Capogrosso M**, **Formento E**, **Wenger N**, **DiGiovanna J**, **Courtine G**, **Micera S**. Mechanisms Underlying the Neuromodulation of Spinal Circuits for Correcting Gait and Balance Deficits after Spinal Cord Injury. *Neuron* 89: 814–828, 2016.

85. **Morimoto T**. Role of electrical activity of neurons for neuroprotection. 1st ed. Elsevier Inc.

86. **Moritz C**, **Perlmutter S**, **Fetz E**. Direct control of paralysed muscles by cortical neurons. *Nature* 456: 639–642, 2008.

87. **Moritz CT**, **Lucas TH**, **Perlmutter SI**, **Fetz EE**. Forelimb movements and muscle responses evoked by microstimulation of cervical spinal cord in sedated monkeys. *J Neurophysiol* 97: 110–20, 2007.

88. **Morris RG**. D.O. Hebb: The Organization of Behavior, Wiley: New York; 1949. [Online]. *Brain Res Bull* 50: 437, 1999. http://www.ncbi.nlm.nih.gov/pubmed/10643472.

89. **Moss CW**, **Kilgore KL**, **Peckham PH**. A novel command signal for motor neuroprosthetic control. *Neurorehabil Neural Repair* 25: 847–854, 2011.

90. **Mothe AJ**, **Tator CH**. Review of transplantation of neural stem/progenitor cells for spinal cord injury. *Int J Dev Neurosci* 31: 701–13, 2013.

91. **Mushahwar V**, **Gillard D**, **Gauthier M**, **Prochazka A**. Intraspinal micro stimulation generates locomotor-like and feedback-controlled movements. *IEEE Trans neural Syst Rehabil Eng* 10: 68–81, 2002.

92. **Mushahwar VK**, **Collins DF**, **Prochazka A**. Spinal Cord Microstimulation Generates Functional Limb Movements in Chronically Implanted Cats. *Exp Neurol* 163: 422–429, 2000.

93. **Mushahwar VK**, **Gillard DM**, **Gauthier MJA**, **Member A**, **Prochazka A**, **Member S**. Intraspinal Microstimulation Generates Movements. 10: 68–81, 2002.

94. **Mushahwar VK**, **Gillard DM**, **Gauthier MJA**, **Prochazka A**. Intraspinal microstimulation generates locomotor-like and feedback-controlled movements. *IEEE Trans Neural Syst Rehabil Eng* 10: 68–81, 2002.

95. **Mushahwar VK**, **Horch KW**. Selective activation and graded recruitment of functional muscle groups through spinal cord stimulation. [Online]. *Ann N Y Acad Sci* 860: 531–5, 1998. http://www.ncbi.nlm.nih.gov/pubmed/9928355.

96. **Mushahwar VK**, **Horch KW**. Muscle recruitment through electrical stimulation of the

lumbo-sacral spinal cord. *IEEE Trans Rehabil Eng* 8: 22–29, 2000.

97. **Nagoshi N**, **Okano H**. Applications of induced pluripotent stem cell technologies in spinal cord injury. *J Neurochem* 141: 848–860, 2017.

98. **Nishimura Y**, **Perlmutter SI**, **Eaton RW**, **Fetz EE**. Spike-Timing-Dependent Plasticity in Primate Corticospinal Connections Induced during Free Behavior. *Neuron* 80: 1301–9, 2013.

99. **Nishimura Y**, **Perlmutter SI**, **Fetz EE**. Restoration of upper limb movement via artificial corticospinal and musculospinal connections in a monkey with spinal cord injury. *Front Neural Circuits* 7: 57, 2013.

100. **NSCISC**. Spinal Cord Injury Facts and Figures at a Glance. *Birmingham, Alabama Natl Spinal Cord Inj Stat Cent* ., 2016.

101. **Nutt SE**, **Chang E-A**, **Suhr ST**, **Schlosser LO**, **Mondello SE**, **Moritz CT**, **Cibelli JB**, **Horner PJ**. Caudalized human iPSC-derived neural progenitor cells produce neurons and glia but fail to restore function in an early chronic spinal cord injury model. *Exp Neurol* 248: 491–503, 2013.

102. **Ogawa Y**, **Sawamoto K**, **Miyata T**, **Miyao S**, **Watanabe M**, **Nakamura M**, **Bregman BS**, **Koike M**, **Uchiyama Y**, **Toyama Y**, **Okano H**. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. *J Neurosci Res* 69: 925–33, 2002.

103. **Pikov V**, **Bullara L**, **McCreery DB**. Intraspinal stimulation for bladder voiding in cats before and after chronic spinal cord injury. *J Neural Eng* 4: 356–368, 2007.

104. **Pinter MM**, **Gerstenbrand F**, **Dimitrijevic MR**. Epidural electrical stimulation of posterior structures of the human lumbosacral cord: 3. Control Of spasticity. *Spinal Cord* 38: 524–531, 2000.

105. **Pizzorusso T**, **Medini P**, **Berardi N**, **Chierzi S**, **Fawcett JW**, **Maffei L**. Reactivation of ocular dominance plasticity in the adult visual cortex. *Science* 298: 1248–51, 2002.

106. **Pizzorusso T**, **Medini P**, **Landi S**, **Baldini S**, **Berardi N**, **Maffei L**. Structural and functional recovery from early monocular deprivation in adult rats. *Proc Natl Acad Sci U S A* 103: 8517–22, 2006.

107. **Ranck JB**. Which elements are excited in electrical stimulation of mammalian central nervous system: a review. *Brain Res* 98: 417–440, 1975.

108. **Rejc E**, **Angeli CA**, **Bryant N**, **Harkema S**. Effects of stand and step training with epidural stimulation on motor function for standing in chronic complete paraplegics. *J Neurotrauma* 16: 1–16, 2016.

109. **Rejc E**, **Angeli C**, **Harkema S**. Effects of lumbosacral spinal cord epidural stimulation for standing after chronic complete paralysis in humans. *PLoS One* 10: 1–20, 2015.

110. **Saigal R**, **Renzi C**, **Mushahwar VK**. Intraspinal microstimulation generates functional movements after spinal-cord injury. *IEEE Trans Neural Syst Rehabil Eng* 12: 430–440, 2004.

111. **Sayenko DG**, **Angeli C**, **Harkema SJ**, **Edgerton VR**, **Gerasimenko YP**. Neuromodulation of evoked muscle potentials induced by epidural spinal-cord stimulation in paralyzed individuals. *J Neurophysiol* 111: 1088–99, 2014.

112. **Sayenko DG**, **Atkinson D a.**, **Dy CJ**, **Gurley KM**, **Smith VL**, **Angeli C a.**, **Harkema SJ**, **Edgerton VR**, **Gerasimenko YP**. Spinal segment-specific transcutaneous stimulation differentially shapes activation pattern among motor pools in humans. *J Appl Physiol* 118: 1364–1374, 2015.

113. **Sayenko DG**, **Atkinson DA**, **Floyd TC**, **Gorodnichev RM**, **Moshonkina TR**, **Harkema SJ**, **Edgerton VR**, **Gerasimenko YP**. Effects of paired transcutaneous electrical stimulation delivered at single and dual sites over lumbosacral spinal cord. *Neurosci Lett* 609: 229–234, 2015.

114. **Sayenko DG**, **Nguyen R**, **Hirabayashi T**, **Popovic MR**, **Masani K**. Method to Reduce Muscle Fatigue During Transcutaneous Neuromuscular Electrical Stimulation in Major Knee and Ankle Muscle Groups. *Neurorehabil Neural Repair* 29: 722–733, 2015.

115. **Schaal SM**, **Kitay BM**, **Cho KS**, **Lo TP**, **Barakat DJ**, **Marcillo AE**, **Sanchez AR**, **Andrade CM**, **Pearse DD**. Schwann cell transplantation improves reticulospinal axon growth and forelimb strength after severe cervical spinal cord contusion. [Online]. *Cell Transplant* 16: 207–28, 2007. http://www.ncbi.nlm.nih.gov/pubmed/17503734.

116. **Schallert T**, **Fleming SM**, **Leasure JL**, **Tillerson JL**, **Bland ST**. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. [Online]. *Neuropharmacology* 39: 777–87, 2000. http://www.ncbi.nlm.nih.gov/pubmed/10699444.

117. **Schwab ME**. Nogo and axon regeneration. *Curr Opin Neurobiol* 14: 118–124, 2004.

118. **Scott CE**, **Wynn SL**, **Sesay A**, **Cruz C**, **Cheung M**, **Gomez Gaviro M-V**, **Booth S**, **Gao B**, **Cheah KSE**, **Lovell-Badge R**, **Briscoe J**. SOX9 induces and maintains neural stem cells. *Nat Neurosci* 13: 1181–9, 2010.

119. **Shah PK**, **Sureddi S**, **Alam M**, **Zhong H**, **Roy RR**, **Edgerton VR**, **Gerasimenko Y**. Unique Spatiotemporal Neuromodulation of the Lumbosacral Circuitry Shapes Locomotor Success after Spinal Cord Injury. *J Neurotrauma* 33: 1709–23, 2016.

120. **Shain W**, **Spataro L**, **Dilgen J**, **Haverstick K**, **Retterer S**, **Isaacson M**, **Saltzman M**, **Turner JN**. Controlling cellular reactive responses around neural prosthetic devices using peripheral and local intervention strategies. *IEEE Trans Neural Syst Rehabil Eng* 11: 186–188, 2003.

121. **Shannon R V.** A Model of Safe Levels for Electrical Stimulation. *IEEE Trans Biomed Eng* 39: 424–426, 1992.

122. **Sharpe AN**, **Jackson A**. Upper-limb muscle responses to epidural, subdural and intraspinal stimulation of the cervical spinal cord. *J Neural Eng* 11: 16005, 2014.

123. **Shealy CN**, **Mortimer JT**, **Hagfors NR**. Dorsal column electroanalgesia. *J Neurosurg* 32: 560–4, 1970.

124. **Shealy CN**, **Mortimer JT**, **Reswick JB**. Electrical Inhibition of Pain by Stimulation of the Dorsal Columns: Preliminary Clinical Report. *Anesth Analg* 46: 489–491, 1967.

125. **Soleman S**, **Filippov MA**, **Dityatev A**, **Fawcett JW**. Targeting the neural extracellular matrix in neurological disorders. *Neuroscience* 253: 194–213, 2013.

126. **Starkey ML**, **Bartus K**, **Barritt AW**, **Bradbury EJ**. Chondroitinase ABC promotes compensatory sprouting of the intact corticospinal tract and recovery of forelimb function following unilateral pyramidotomy in adult mice. *Eur J Neurosci* 36: 3665–78,

2012.

127. **Stokes BT**, **Reier PJ**. Fetal grafts alter chronic behavioral outcome after contusion damage to the adult rat spinal cord. [Online]. *Exp Neurol* 116: 1–12, 1992. http://www.ncbi.nlm.nih.gov/pubmed/1559561.

128. **Sunshine MD**, **Cho FS**, **Lockwood DR**, **Fechko AS**, **Kasten MR**, **Moritz CT**. Cervical intraspinal microstimulation evokes robust forelimb movements before and after injury. *J Neural Eng* 10: 36001, 2013.

129. **Sunshine MD**, **Cho FS**, **Lockwood DR**, **Fechko AS**, **Kasten R**, **Moritz CT**. Cervical intraspinal microstimulation evokes robust forelimb movements before and after injury. *J Neural Eng* 10, 2013.

130. **Tan AM**, **Chakrabarty S**, **Kimura H**, **Martin JH**. Selective corticospinal tract injury in the rat induces primary afferent fiber sprouting in the spinal cord and hyperreflexia. *J Neurosci* 32: 12896–908, 2012.

131. **Tator CH**, **Minassian K**, **Mushahwar VK**. Spinal cord stimulation: therapeutic benefits and movement generation after spinal cord injury. *Handb Clin Neurol* 109: 283–96, 2012.

132. **Tester NJ**, **Howland DR**. Chondroitinase ABC improves basic and skilled locomotion in spinal cord injured cats. *Exp Neurol* 209: 483–96, 2008.

133. **Tetzlaff W**, **Okon EB**, **Karimi-Abdolrezaee S**, **Hill CE**, **Sparling JS**, **Plemel JR**, **Plunet WT**, **Tsai EC**, **Baptiste D**, **Smithson LJ**, **Kawaja MD**, **Fehlings MG**, **Kwon BK**. A systematic review of cellular transplantation therapies for spinal cord injury. *J Neurotrauma* 28: 1611–82, 2011.

134. **Tsuji O**, **Miura K**, **Okada Y**, **Fujiyoshi K**, **Mukaino M**, **Nagoshi N**, **Kitamura K**, **Kumagai G**, **Nishino M**, **Tomisato S**, **Higashi H**, **Nagai T**, **Katoh H**, **Kohda K**, **Matsuzaki Y**, **Yuzaki M**, **Ikeda E**, **Toyama Y**, **Nakamura M**, **Yamanaka S**, **Okano H**. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. *Proc Natl Acad Sci U S A* 107: 12704–9, 2010.

135. **Ward AR**. Electrical Stimulation Using Kilohertz- Frequency Alternating Current. *Phys Ther* 89: 181–190, 2009.

136. **Ward AR**, **Shkuratova N**. Russian Electrical Stimulation: The Early Experiments. *Phys Ther* 82: 1019–1030, 2002.

137. **Zhang J**, **Calafiore M**, **Zeng Q**, **Zhang X**, **Huang Y**, **Li R a**, **Deng W**, **Zhao M**. Electrically guiding migration of human induced pluripotent stem cells. *Stem Cell Rev* 7: 987–96, 2011.

138. **Zhao RR**, **Andrews MR**, **Wang D**, **Warren P**, **Gullo M**, **Schnell L**, **Schwab ME**, **Fawcett JW**. Combination treatment with anti-Nogo-A and chondroitinase ABC is more effective than single treatments at enhancing functional recovery after spinal cord injury. *Eur J Neurosci* 38: 2946–2961, 2013.

139. **Zhao RR**, **Muir EM**, **Alves JN**, **Rickman H**, **Allan AY**, **Kwok JC**, **Roet KCD**, **Verhaagen J**, **Schneider BL**, **Bensadoun JC**, **Ahmed SG**, **Yáñez-Muñoz RJ**, **Keynes RJ**, **Fawcett JW**, **Rogers JH**. Lentiviral vectors express chondroitinase ABC in cortical projections and promote sprouting of injured corticospinal axons. *J Neurosci Methods* 201: 228–238, 2011.

140. **Zimmermann JB**, **Seki K**, **Jackson A**. Reanimating the arm and hand with

intraspinal microstimulation. *J Neural Eng* 8: 54001, 2011.